CONNECTIVE TISSUES

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THE JOSIAH MACY, JR. FOUNDATION CONFERENCE PROGRAM

WHEN I was on a destroyer out at Bikini in 1946 I was fascinated listening to our radio operator as he tested communication equipment. He would ask another ship through his radio, "How do you hear me?" and the answer often would come back, "I hear you Nine-Nine-Nine." That meant that everything was satisfactory. Of the three nines, one was for intensity, one for clarity, and one for meaning.

The Josiah Macy, Jr. Foundation has organized and devoted a large portion of its resources to the support of its Conference Program because the officers are cognizant of the fact that there is considerable obstruction to communication and mutual understanding across the disciplines and specialties, and that this, in fact, is one of the major factors delaying scientific advance. We feel that there are psychological as well as semantic factors contributing to the difficulty of communication, people, even in arguments with one another, are too much inclined to make statements *at*, rather than to communicate *with*, others. I think that we are inclined to forget, though, that the real question is, are these words and statements those which are likely to convey to the listener the whole or even a small part of what I would like to express.

I have a feeling that we should be very much concerned with the other fellow's receiving set and not only with our own transmitter. If the other person doesn't seem to understand us, it may not be enough merely to increase the power of our transmission, we must try to find the obstruction in his receiving set, and see what kind of filters and resistors he uses. So, if we call out to the interprofessional No-Man's-Land, "How do you hear me?" and the reply comes back, "I hear you Nine-Nine-Nine," we have the beginning of communication. What we try to do in these conferences conducted by the Foundation is to set the stage for meaningful communication.

With the accelerating rate at which new knowledge is accumulating and with the increasing recognition that nature is of one piece, it becomes evident that the continued isolation of the several branches of science from one another is a serious obstacle to scientific progress. Nowhere in science is the need for "combined operations" more evident than in medicine. Today, to be effective, medical research and practice must embrace data from all the disciplines including nuclear physics at one end of the spectrum and cultural anthropology at the other, for

advances in one field are frequently dependent upon knowledge derived from quite another discipline

Although the fertility of the multidiscipline approach is thus recognized, universities, and scientific societies and journals which are usually restricted to one small area of a field in their coverage, have not yet made adequate provision for channels of interdisciplinary communication. We do not wish to compete with the formal scientific meetings or with the scientific journals which have established patterns and formats for the presentation of material. Our purpose at the meetings is to keep an informal atmosphere and to encourage the exchange of methods, research plans, concepts and difficulties, which cannot be done if there is formal speech making

The Foundation has endeavored to meet the need for interdisciplinary communication by bringing together for a series of two-and-a-half day annual conferences a small group of investigators, representing in so far as possible all the branches of science related to a chosen problem Participants in these informal conferences over a five-year period develop a feeling of friendship, trust and mutual respect which in turn promotes communication, cross fertilization of ideas and cooperation The success of such an endeavor, however, is dependent upon full participation of all members in the discussion Accordingly attendance at any conference is limited to twenty-five.

Under the guidance of Dr Willard C Rappleye, President of the Foundation since 1942, the Conference Program has been gradually expanded and enlarged until during 1953 it included twelve different groups which meet annually to discuss a wide variety of problems in the field of medicine and the closely related disciplines Our plan is to discontinue the meetings of each group at the end of five years.

In order to share with a wider group of investigators and students the essential quality of these conferences and to give others an insight into the functions of the scientific mind, the informal nature and tempo of the discussions, as far as possible, are preserved in the published transactions

FRANK FREMONT-SMITH, M.D
Medical Director

INTRODUCTORY REMARKS

Ragan I think it might be interesting to hear what each of you is thinking and doing, and I shall ask Dr Angevine to begin the proceedings

Angevine. I am sorry to say that I am concerned with the direction of research rather than with actual participation in it It is one of the sadder commentaries on American medicine today, that people who start as investigators soon find themselves administering rather than carrying out experiments with their own hands

I think even this conference indicates that we have become more peripatetic than we should However, I work on the basis that I will refuse many of the commitments that come my way and accordingly have become less peripatetic every year Perhaps science would progress little better if more of us remained at home and associated with the young people who are actually doing the work Therefore, what I have to say is really what is going on in my department, rather than what I am doing myself

As this conference has brought out, new approaches are needed to the study of connective tissues We are working, at present, with the disease known as lathyrism, which as most of you probably know is concerned with the effect of sweet peas on various animals, in other words, if one feeds sweet pea seed to a rat, the animal will develop a disease with some similarities to osteoporosis in man, or something which very closely parallels it This work is being done by Dr J J Lalich, Dr Frank M Strong, and Mr Gerald McKay

You may ask, "Why do you work on that?" We are interested in it because it produces a disease of the bones, osteoporosis, and secondly, it produces aneurysms of the aorta Several experimenters, who have attempted to repeat some of the work on aneurysm of the aorta, have been unsuccessful, but that is because they have not followed precisely the methods of Ponseti and Baird (1) We have confirmed this observation

Now, what are we doing? In the first place, we are attempting to define the alterations in the connective tissue matrix prior to calcification in normal and abnormal situations in these rats We are doing this, first, by utilizing histochemical methods, which, as we know, are crude at best We are also attempting to study the changes in the density of cartilaginous matrix by historadiography, which, as I have indicated before, is a very useful tool in histochemistry, and in some instances has

shown us that certain histochemical methods have been rather misleading.

Because these rats develop both osteoporosis and aneurysms, we feel that this disease is primarily one of the connective tissues Dr J. J. Lalich, in our department at the University of Wisconsin Medical School, has been attempting to determine the amino acid patterns in the aorta of control and treated rats, and he believes there is a difference in the two groups which suggests that there may be some basic disturbance at work

Ragan Is Dr Lalich studying serum?

Angevine No, only tissue

Ragan: Which tissue?

Angevine He has been working only on the total aorta, but plans to do studies on the skeleton later on.

Gyorgy. Does he use hydrolyzed protein? What do you mean by amino acids?

Angevine He is working with an extract of the entire aorta, and is attempting to obtain a paper electrophoretic pattern on that In other words, he has not attempted to break it down into various components

Gyorgy How does he obtain the amino acids?

Angevine They are hydrolyzed.

Ragan· Is this done before the rats are put on the diet?

Angevine These diseases were produced in young rats, it was done after they have been on the diet for about three to four weeks

Ragan. Before they developed the aneurysm?

Angevine Yes If kept on the diet, chronic changes may be produced, which take about twelve to fourteen weeks.

One of the reasons we have been especially interested in this problem is that Dr Frank M Strong, of the Department of Agricultural Biochemistry, at the University of Wisconsin, has been successful in isolating from the sweet pea the toxic factor which produces the lesion All I can say at present is that it is a dipeptide and that we probably will be able to find a similar substance on our chemical shelves

Gyorgy Is the dipeptide administered by mouth, or by injection?

Angevine We have administered it by mouth. Dr. Strong (2) will report at Atlantic City in April, 1954, on the exact nature of the substance

Ragan. What do you use as your end point?

Angevine: As to production of a lesion?

Ragan Yes, the aortitis, or the osteoporosis?

Angevine The changes are shown by x-ray, and in addition to these

changes, which are very definite, the vertebral columns of these rats almost resemble the letter "S" they become so deformed As far as we know, the calcium and phosphorus metabolism, and the calcium phosphorus ratio of the bone ash, are normal, so we feel that the defect is probably in the connective tissue There are definite changes in the cartilage, I have sent sections to Dr R H Follis, Dr Granville Bennett, and Dr Burt Wolbach, to see what they think of the pathological changes, which have not been very well described

Ragan I thought Ponseti (1) did fairly conclusive studies on that

Angevine He described the changes in the bone, which anyone can see with the naked eye, but I do not believe he has gone very deeply into the pathogenesis of the lesion We are not entirely sure whether the paralysis, which these animals develop, is due to alterations in the vertebrae, or whether there is still a toxic factor for the central nervous system. There may be two factors

Meyer I thought that Ponseti's paper in the *American Journal of Pathology* stated that all the changes in the nervous system, or in the spinal cord, were secondary to slipped discs or actual fractures of the vertebrae.

Angevine I do not think that is so

Ragan There is some difference of opinion on that

Gyorgy Clinically, it must be different

Angevine That is right We have done a few myelograms and have not been able to corroborate it, further studies are necessary We are principally concerned as to why these changes occur very early I think that is the crux of the situation

Ragan They are malnourished rats, are they not?

Angevine That is an interesting point, Dr Ragan, they are not malnourished rats until the chronic stage is reached

Ragan How about the weight gain?

Angevine There is no marked change in weight, they are fair healthy rats

Ragan Ours were also healthy rats, but they had not gained muc weight. They were still very young rats when they were put on th diet.

Angevine Except for roughness of the hair, the only change in the appearance of the rats is that they are shorter because of the vertebral column deformation

Meyer When do you start feeding them?

Angevine We start at about three or four weeks They are very young rats to start with and have continued to use some of them for about eight to ten months

Ragan We do the same.

Angevine Our interest in this work has been largely because of the fact that the toxic factor has been isolated, which I think may help us to understand some of these conditions.

I shall mention briefly a few of the other things that are going on in our department at the University of Wisconsin. I am not the one who is doing the work, and that is probably why I am not answering all the questions Mr Don Churchill is investigating the alterations in the ground substance of the central nervous system during injury. People have described the ground substance of the central nervous system, but very little attention has been paid to it during injury Another important current project is that which I discussed in some detail last year it concerns the muscle function and structure in relation to enzyme activity, on which Dr J W Harman has been working.

Ragan Where is the ground substance of the central nervous system?

Angevine In young animals it is diffusely located between the nerve fibers, and there is a considerable amount of it. When the animals are fully developed, one sees far less The bulk of it, of course, as one would expect, is in the meninges, and in the perivascular tissues or blood vessels

Ragan Is it metachromatic? Is it PAS-positive?

Angevine Yes, it is We have been able to confirm the work that has been reported from Dr Dempsey's laboratory (3) We are chiefly concerned now with what happens during injury, rather than with the fact that it is still present

Ragan: Does fibroplasia follow injury in the central nervous system?

Angevine We haven't enough evidence to answer that question

Ragan Dr Travell, will you continue?

Travell As you know, we have been studying mechanisms of pain arising in myofascial tissues This is also the work of a group,* and I wish to mention especially the collaboration of Dr Seymour H Rinzler, of Cornell University Medical College, Dr. Hyman Bakst, of Beth Israel Hospital, New York, and more recently, Dr. Isidore Stein, of Coney Island Hospital, Brooklyn.

We have been concerned mainly with three questions: First, what is the nature of the peripheral focus known as the "trigger area," which mediates pain impulses from the musculoskeletal structures, and maintains a continuing pain cycle? The second question is, what are the

*These investigations have been supported (in part) by the Josiah Macy, Jr Foundation, and the National Heart Institute of the National Institutes of Health, Public Health

relationships of myofascial trigger mechanisms to pain referred from diseased viscera? And the third question is, what is the mechanism by which local procaine infiltration (or a brief application of a rapidly cooling spray such as ethyl chloride or a mixture of freons) interrupts the peripheral focus and pain cycle?

With respect to the first question, the nature of the trigger area, we have encountered a number of misconceptions as to what is meant by the term "trigger area." For the benefit of our visitors who have not been present at previous discussions, I should like to say that a trigger area is not a disease, a diagnosis, a symptom, or something that the patient complains of If he complains of pain, it is usually not at the site of a trigger area

Actually, a trigger area is a physical sign It is discovered on physical examination, and its presence is revealed by three things. first, circumscribed, deep tenderness, with a lowered deep pain threshold as measured by the algesiometer, secondly, a localized twitch or fasciculation, when stimulated by pressing or pinching, of that portion of the muscle which contains the trigger area, which is usually both a palpable and a visible sign, and thirdly, the description by the patient of the appearance of pain at a distance from the trigger area, when this is stimulated by pressure

We have not so far obtained a definitive answer as to what precisely a trigger area is, in terms of pathophysiological processes, but we have made some interesting observations In a number of patients we inserted a needle thermocouple into a trigger area with the idea of determining whether the temperature at that point was reduced, as one might anticipate if it represented an area of vasoconstriction and reduced blood flow, or whether the temperature was increased, as would be the case if it represented an area of vasodilation

Our findings have shown that when the needle thermocouple is inserted into a trigger area, the temperature appears to be elevated, since the initial readings during the first 15 to 60 seconds are higher than the subsequent ones During the first half minute or so, the temperature curve falls off by a quarter to a half degree centigrade, and then levels off We have not seen the temperature rise following the insertion of the needle thermocouple into a trigger area in a muscle

Of course, when the needle hits the trigger area, a transient reference of pain is set off Fading out of this induced pain seems to coincide with the leveling off of the temperature in the trigger area

We do not necessarily take this to mean that there is a state of vasodilation in the trigger area It is possible, rather, that the temperature elevation is due to increased muscle work, that is, to a steady state of

contraction of that portion of the muscle which contains the trigger area and which appears to be in spasm

Ragan Do you obtain action potentials in the muscle?

Travell The patient feels tense to palpation We have not been able to run any electromyographic recordings simultaneously with our temperature recordings We had formerly made a few observations, using surface electrodes, and there appeared to be a continuous small activity at the site of a trigger area. We did not think a needle electrode would be satisfactory for recording, because the minute a needle is inserted squarely into a trigger area, it disappears

Fremont-Smith When you put a needle into the trigger zone, do the surface recording action currents disappear?

Travell We actually have not done that experiment. We were simply comparing demonstrable trigger areas with muscle areas that were not tender and contained no trigger areas

Ragan We have done work on rheumatoid muscles, with flexion contractures; when the joint is not extended beyond pain limits, but still with a flexion contracture, there are no action potentials in the muscles It is only when it is extended beyond pain limits, and the patient starts to have pain, that the action potentials appear

Travell Wouldn't the stretching of the muscle increase the internal pressure within the muscle?

Ragan· I do not know These muscles are presumably in spasm, because there is a contracture which holds them in flexion, but they are not in spasm as far as actual electrical potentials are concerned

Fremont-Smith Until they are stretched to the pain point?

Ragan Beyond the pain point

Travell Observations have been made on painful muscles thought clinically to be in spasm, in which no action potentials were demonstrated Harell (4) discusses this question.

Ragan A muscle cannot be in spasm if it does not have an electrical potential, can it?

Travell It can resist stretching, but it may not contract actively until then

Fremont-Smith It is a question of the definition of "spasm." If we define it as something which does have electrical potentials, then obviously the absence of electrical potentials eliminates spasm I am inclined to agree that if we do not have action potentials, it should not be called spasm But I think it is our use of the term that leads us into this kind of difficulty.

Ragan To me, at least, spasm mean[illegible] ng active, and if we [illegible] is at rest

Holbrook. But you're beating around the bush there. These muscles are shortened

Travell. They will not stretch

Holbrook. That is the point.

Ragan. That is perfectly true, but they are still not in spasm

Holbrook. Of course not until they are pulled But they shorten, and then when they are pulled they go into spasm But they will undergo progressive shortening without spasm I agree with that

Travell. While at rest, they would not necessarily show action potentials

Ragan. But then, by that definition they are not in spasm

Holbrook. Of course not

Travell. They do not go into spasm until they are stretched or touched with a needle, which actually initiates a twitch or a localized contraction of that portion of the muscle

Ragan. That happens to any muscle when it is touched with a needle

Travell. No, in our experience it does not We insert a needle into many muscle areas and neither induce a muscle contraction, nor set off a pain sensation There will be no change in the level of the muscle temperature from the initial resting temperature, unless perhaps there may be a slow, slight rise probably due to hyperemia of tissue injury It is surprising how many areas in healthy muscle can be riddled with needles, without complaint from the patient, and without anything happening

Fremont-Smith When you insert a needle into normal muscle, have you ever observed whether you do or do not evoke any action currents?

Travell We were not primarily studying normal muscles

Fremont-Smith. Was your work done with action currents, Dr Ragan?

Ragan Yes, using an electromyograph

Travell Of course, when normal muscles are injured by a needle, we do not obtain a localized contraction of the muscle, as we do when we touch a trigger area

Fremont-Smith It may be, again, a question of what you mean by "contraction" You may mean a fasciculation of a group of fibrils, and Dr Ragan may mean an electromyographic evidence of muscle activity

Ragan Exactly, any time we insert a needle into a muscle we are damaging it, in my opinion

Travell Yes, but that may not initiate a gross muscle contraction

Ragan I'm talking about electromyographic evidence, or an action

Ragan: What does ergonovine have in it?

Travell: Ergonovine maleate is a synthetic alkaloid related to ergotamine and other ergot alkaloids. Ergonovine, however, does not produce the tachycardia and the rise in blood pressure which accompany the injection of ergotamine

Figure 1 shows a study on a patient who had angina of effort. A dose of 0 1 mg. of ergonovine was injected intravenously, and within one minute depression of the S-T segment was evident in Lead II. At the same time, pain appeared in the elbows and in the neck, the same areas where the patient developed pain on walking. After about six minutes, ethyl chloride spray was applied in sequence to the different pain areas, and for each one in turn, pain disappeared within fifty seconds On the other hand, the S-T depression persisted, and even increased until nitroglycerin was given. Only then did the electrocardiogram return to normal.

Figure 2 illustrates what happens when ethyl chloride spray is applied *before* the ergonovine is injected. In the first or control ergonovine

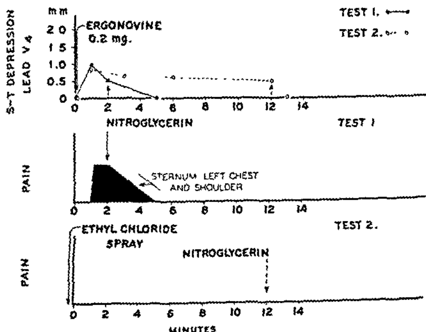


FIGURE 2 Effect of prior spraying with ethyl chloride (Test 2), on ergonovine-induced pain and S-T [illegible] effect of ergonovine alone (Test 1), in A. F., a 61-year-old [illegible] changes were not influenced by [illegible] H., Stein, I., Bakst, H., Weinstein [illegible] ethyl chloride spray on cardiac pa[illegible] Med 85, 329 (1954)

test, chest pain and S-T depression both appeared in about one minute Nitroglycerin was given in two minutes, and about three minutes later the electrocardiogram was normal and pain disappeared In the second test, which was done a week later, no pain appeared after the ergonovine injection, which had been immediately preceded by application of ethyl chloride spray. Nevertheless, the electrocardiogram became abnormal in one minute after the ergonovine, and remained abnormal for as long as twelve minutes, when the S-T segment changes were reversed by nitroglycerin

During the first test, the areas in which pain appeared were noted Then at the next visit, just before the ergonovine was injected, each of these predicted pain areas was sprayed for one to two minutes with about six sweeps of the ethyl chloride spray, covering the entire skin area once or twice The effect on skin temperature produced by a single sweep of a rapidly cooling spray is shown in Figure 3 Sometimes we used ethyl chloride spray, and sometimes a mixture of freons with similar physical properties.

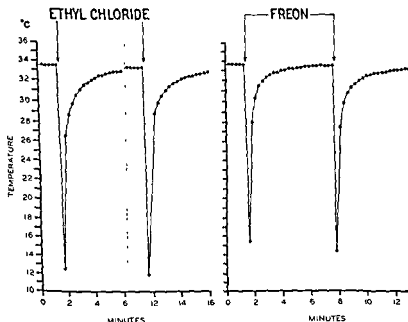


FIGURE 3 Effect of two single sweeps of ethyl chloride, and two of a freon mixture, on temperature of skin surface, as recorded by an iron-constantan thermocouple (Leeds and Northrup Speedomax) Temperature is recorded at 16-second intervals

The ergonovine angina was not always completely prevented by prior application of the spray. Figure 4 shows that in one patient prior spraying of the back, sternum, and shoulder, with ethyl chloride caused a

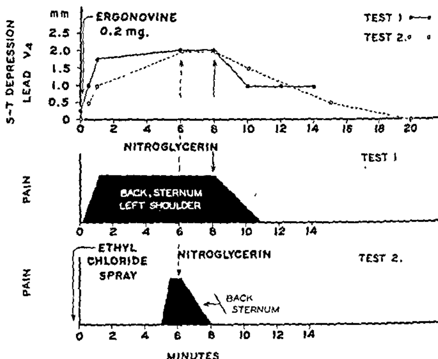


FIGURE 4 Effect of prior spraying with ethyl chloride (Test 2 on ergonovine-induced pain and S-T depression, as compared with effect of ergonovine alone (Test 1), in A P, a 70-year-old female Onset of pain was delayed, while electrocardiographic changes were not influenced by the spray.

five-minute delay in the onset of pain, although the changes in the electrocardiogram progressed just as in the control test with ergonovine alone Both pain and ECG changes were terminated later by nitroglycerin. The actual electrocardiograms taken in these two tests are shown in Figure 5. It may be seen that the S-T segment was depressed to about the same extent after the dose of ergonovine, whether or not the spray was applied.

Table I summarizes the results to date of the control procedures and the ergonovine tests in relation to the application of the spray. Ergonovine by itself evoked pain in all the tests, together with electrocardiographic changes. Control injections of saline were entirely negative. Nitroglycerin, given sublingually just before ergonovine, as a rule prevented both the pain and the electrocardiographic changes, presumably

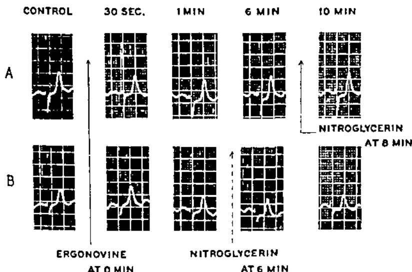


FIGURE 5 Electrocardiograms showing effect of intravenous ergonovine on S-T segment (*A*) without ethyl chloride spray, and (*B*) with prior application of the spray, in the tests illustrated in Figure 4

TABLE I

Influence of a Rapidly Cooling Spray (Ethyl Chloride or Freon) on Pain and Electrocardiographic Changes Induced by Ergonovine in 13 Patients with Effort Angina and a Normal Electrocardiogram

Procedure	Number of Tests	Number Evoking ECG Changes	Number Evoking	
			Pain	No Pain
Ergonovine* alone	15	15	15	0
Ergonovine preceded by cooling spray	15	15	6	9
Ergonovine preceded by nitroglycerin	14	2	1	13
Physiological saline*	14	0	0	14
Ergonovine followed by *cooling spray*†	12	12	Pain Relief: 12 { Partial 10, Complete 2	

†Spray applied after pain and ECG changes had appeared
*Injected intravenously

ed pain areas, pain failed to develop at all after ergonovnie in the majority of cases, that is, in 9 out of 15 tests (60 per cent); in the other 6, pain was delayed in onset, or reduced in intensity. Thus, standardized cardiac pain was blocked by altering the flow of afferent impulses from the somatic reference zone, even while objective signs of cardiac disorder persisted in the electrocardiogram. It seems most likely that the blocking of pain results from a counterirritant action of the spray, and an alteration in the central excitatory state.

Ragan Dr Mirsky, would you like to discuss the effect of cortisone, ACTH, and Compound F, on peptic secretions?

Mirsky: I should like to discuss the statement that the administration of ACTH, cortisone, or Compound F, in the treatment of a variety of clinical states, results in an ulceration of the gastrointestinal tract. The impression has been given that it is a universal response to the use of these drugs.

In 1949, we presented some data (8) to indicate that when patients are exposed to a variety of noxious stimuli, an increase in gastric secretion, as measured by pepsinogen excretion in the urine, may occur in some of them We postulated that perhaps the secretion of ACTH played a role in that phenomenon. Very shortly thereafter, a series of papers began to appear in which evidence was presented that the administration of ACTH, or adrenocortical hormones, resulted in an increase in pepsinogen excretion and in gastric secretion It is now frequently stated that activation of the adrenal cortex can cause a reactivation of ulcers, or the initiation of gastric and duodenal ulcers

In a random study of patients who had received ACTH, cortisone, and Compound F, we could find no differences between the pepsinogen of the blood and urine as compared with a group of patients who had not received these compounds More recently we studied the effects of ACTH, cortisone, and Compound F, on the blood and urinary pepsinogen of a series of healthy subjects After a "control" period of from 12 to 28 days, they were given the hormones for a similar period and then followed for an additional "post-treatment" period. We found no difference between the data obtained in the period during which the subject received hormones, and that obtained prior to, and after, the administration of these hormones.

Ragan Dr Meyer, would you like to continue?

Meyer I jotted down a few points on problems we are working on, and naturally I do not have to tell you that they are chemical problems. First, we have now more or less determined the structure of the disaccharide, produced by bacterial hyaluronidase from hyaluronic acid, and from the partly or completely desulfated chondroitin sulfate. It repre-

sents a new type of carbohydrate which is produced by all the hyaluronidases of bacteria that we have investigated. The disaccharide produced by these bacterial hyaluronidases from hyaluronic acid is composed of an unsaturated uronic acid and hexosamine. The hexosamine is the reducing end, and it is N-acetylated. After hydrolysis, the hexosamine is isolated and has proved to be D-glucosamine. The uronic acid is derived by splitting out from the original glucuronic acid moiety the elements of water.

We assign the following structure to the compound (Figure 6). The double bond is between carbon 4 and 5. It absorbs in the ultraviolet at

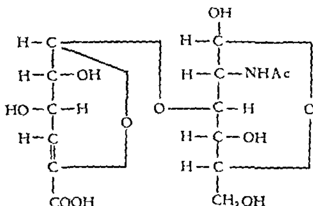


FIGURE 6 Bacterial disaccharide

235 mμ, and takes up one mol of bromine and two mols of hydrogen on catalytic hydrogenation, with palladium catalyst and yields on ozonization oxalic acid.

The normal repeating unit of hyaluronic acid has been shown by Weissmann and Meyer (9) to be β-glucuronido-1-3 N-acetyl glucosamine, which is polymerized in a straight chain presumably also via a 1-3 glycosidic bond. Animal hyaluronidases hydrolyse these hexosaminidic bonds yielding the normal disaccharide, N-acetylhyalobiuronic acid, and multiples of this disaccharide. Since bacterial hylauronidases yield only the unsaturated disaccharide, we have to conclude that every glucosaminidic bond broken leads to a modified nonreducing end-group. We are attempting to find out whether this unsaturated glucuronidic group occurs in nature.

We are also working on other structural problems. One graduate student has been working on the structure of chondrosin, the deacetylated and desulfated repeating unit of chondroitin sulfate of cartilage, and on the sulfated N-acetylated compounds produced by enzymatic di-

gestion We also are continuing work on the nature of the mucopolysaccharides produced by different mesenchymal tissues We have started to work on bone and hope to obtain some information on calcification. In the same program, we are collaborating with Drs. H. Grossfeld, J Decker, and G. Godman, of the Department of Medicine and the Histochemistry Research Laboratory, Columbia University, College of Physicians and Surgeons, on the nature of the polysaccharides produced by mass tissue culture, and hope to study enzyme systems involved in the synthesis. Another project is a collaborative study on the macromolecular structure of hyaluronates as determined by physical means Dr. B Blumberg, Fellow of the Arthritis and Rheumatism Foundation, is working on this phase of the problem.

We are also working on a fraction which we call the hyaluronic acid-like fraction, isolated from cornea Similar fractions probably are present in other tissues. This fraction turned out to be chondroitin This was shown by isolation of the aminosugar, which proved to be chondrosamine Chondroitin behaves in every respect like hyaluronate, it is hydrolysed by both testicular and bacterial hyaluronidases at a rate identical with that of hyaluronate. This is of some importance since, histologically, fractions have been identified as hyaluronate on the basis of their hydrolysis by streptococcal hyaluronidase. The presence of chondroitin suggests that the carbohydrate skeleton is first polymerized, and afterward sulfated This problem is of great importance in the understanding of the results with radiosulfate. The experiments in various laboratories have shown a rapid incorporation of sulfate, and a relatively rapid turnover of organic sulfate. So far as we know, this, in the absence of a sulfatase which is capable of hydrolyzing the carbohydrate sulfate esters, speaks very strongly for the above hypothesis However, it would be futile, at this stage, to speculate on the problem.

Ragan. Dr Gyorgy?

Gyorgy At present, I am concerned with a problem which is related to our topic only from the chemical point of view. I am interested, namely, in the mucopolysaccharides I am a pediatrician and I have been interested in human milk which is the natural food for the human infant, whereas cow's milk is for the calf. I found a microbiological growth factor in human milk which is practically absent from cow's milk, which contains, on an average, approximately 1/40th of the amount present in human milk This growth factor belongs in the group of mucopolysaccharides, ranging from polysaccharides down to relatively small oligosaccharides.

It is quite a spectrum which goes down from the blood substance to these oligosaccharides in breast milk. That led us, of course, to the

study of their chemistry and their biological nutritional role I probably could not give you one per cent of the findings today, but I should like to point out a very few salient facts

This microbiological growth factor is found in human milk, and in a much larger quantity in human colostrum It is found even in the cow's colostrum, yet it is practically absent in late cow's milk. In fact, in many animals it is usually present in much larger amounts in early milk than in late milk. In human milk, it remains on a relatively high level, and is also still higher in the colostrum The growth factor is present in gastric mucosa, duodenal mucosa, and in gastric mucin. There cannot be any doubt about the original assumption, perhaps completely proven by Glass (10), and more recently by Latner (11), that the intrinsic factor is part of this group of mucopolysaccharides with high microbiological activity

Furthermore, the same substance in nutritional dietary studies will promote the growth of animals, when given as a supplement to a diet low in protein In addition, it has lipotropic activity, and will also prevent dietary cirrhosis This is especially interesting in relation to the world-wide problem of malnutrition, which is perhaps the paramount public health problem in the world at large

Low protein intake is a major cause of protein malnutrition in tropical countries Human milk is very low in protein; it contains about 1 2 per cent. Therefore, it is difficult to understand why human milk is one of the best preventives for protein malnutrition Although it has the lowest protein content of any milk, nevertheless it will still, without a doubt, prevent protein malnutrition I am now working on the hypothesis that these groups of mucopolysaccharides, and related substances, are able to spare protein

Finally, the same group of polysaccharides, oligosaccharides, disaccharides, or carbohydrates, with a not quite well-determined structure, are somehow apparently related to antiviral activity They have this activity, (as we have also shown for fractions obtained from human milk) by interfering with the growth and multiplication of the virus within the cell. It seems obvious that this will open—perhaps optimistically speaking—quite a large field of natural resistance, if we may call it that, or at least resistance imparted by nutrition

As I mentioned earlier, this is related to the connective tissue field only because it opens up very wide, and hitherto neglected, field of chemical compounds, with which modern medicine will have to deal, I believe, during the next decade

Meyer I think there is a closer relation between the work of your group and connective tissues, Dr Gyorgy I should like to remind you

of the N-acetyl glucosamine-galactoso-sulfate mucopolysaccharide recently described by our laboratory. Then there are hexosamine-galactose complexes which, combined with lipids, are probably constituents of all cell membranes. Further, some of the serum mucoid fractions contain hexosamine, galactose, mannose and fucose. Recently, a paper was published from Leblond's (12) laboratory in which, by paper chromatography, galactose, glucose and fucose were shown in digests of reticular tissue Unfortunately, no quantitative data were reported, but it seems likely that galactose, possibly combined with hexosamine, and perhaps mannose, occurs as a mucoid in connective tissue, just as Masamune and his group (13) in Japan have long maintained.

It seems to me extremely interesting that the disaccharide units you described are growth factors Do you believe that these units start the process of polymerization to polysaccharides in the tissue?

Gyorgy We are a little far away from an answer at the present time. Unquestionably, the galact[illegible] charide appears to me very [illegible] systems under consideratio[illegible] kind of prosthetic group, with the difference that it may polymerize, which would be unusual for other prosthetic groups. But the disaccharide that we found, and which can be synthesized enzymatically, is the most active microbiological growth factor for the special strain we are working on, and therefore we call it the basic unit

What in the long run it will mean, I do not know I know it is not produced in the liver, because the liver is very poor in this microbiological growth factor It is practically free, I should say. Only goblet cells produce it, we found that where goblet cells exist, this growth factor is present The circulating serum, mucoprotein, is not active as a microbiological growth factor, perhaps because it lost it through polymerization, or by complex formation The proper protein of this very unusual disaccharide, containing acetyl glucosamine and galactose in the higher molecular compound, still has to be elucidated But I think it plays a very important biological role.

Meyer You do not know whether the disaccharide occurs in phosphorylated form?

Gyorgy We are working on it My original idea has been that since it is present in human milk, which has the highest lactose content of any milk, it might have to do with the metabolism of galactose and lactose Therefore, I have been thinking of Caputto and Leloir (14, 15) and their coenzymes But we do not find any uridine phosphate hexosamine in milk, and we not know whether they are interrelated We find it, of

course, in the liver. My feeling is [illegible] it has something to do with the metabolism of galactose

Fremont-Smith. Dr Gyorgy, ar[illegible] goblet cells in the gland cells of the breast?

Gyorgy. In the duct, I believe [illegible] right, Dr Angevine?

Angevine: If there are any the[illegible] in the duct, but there must be very few of them Of course, we see [illegible] droplets in these cells. I have always assumed that goblet cells are not present in the breast.

Gyorgy: I do not know whether they are produced in the breast.

Fremont-Smith But they are in the milk?

György Yes

Fremont-Smith They are much more highly concentrated in the colostrum, if I understand you rightly. Is it possible that in the very first few days, when the colostrum is formed, there is a larger number of goblet cells and this might disappear?

György: I do not know

Fremont-Smith This could be studied in the animal A comparison could be made between the time of parturition when the nursing begins and the situation a week later, in the terms of the incidence of goblet cells

György. One of the most puzzling problems, from the biological [illegible] chemical point of view, is that this factor, which acts as a microbiol[illegible] cal growth factor, may be replaced by a very high molecular compo[illegible] such as pure or highly purified blood group substance, with a molecu[illegible] weight of 260,000 This material came from Prof Walter T J M[illegible] of London, England, he considered it homogeneous H[illegible] bacteria respond to such a high molecular substance? I have bee[illegible] with the idea that these bacteria contain some enzymes, and the[illegible] own the high molecular compound before the substance [illegible] urned out that this specific strain of *Lactobacillus bifidus* [illegible] this factor for its growth, contains an enzyme which com[illegible] vates the growth factor It is difficult to explain why [illegible] essential for growth, yet is made inactive by the enzy[illegible] strain.

Breast milk is very rich in this factor Therefore, we [illegible] tissue, hoping that it would break down blood group su[illegible] in the milk, as I mentioned before, we have high mole[illegible] very low molecular fractions We did not find the c[illegible] which it would break it down Therefore, we are dealin[illegible] lem of whether bacteria can utilize very high molecula[illegible] growth

[illegible] Before accepting the hypothesis [illegible]

cular weight of 200,000 is utilized as such, I would make very sure that such a substance is *not hydrolyzed by the bacteria.*

Gyorgy I just have not yet found the condition, although I have tried many things.

Holbrook: Dr Gyorgy, you said that this substance was forty times as concentrated in human milk as in cow's milk, did you not?

Gyorgy Yes, in late cow's milk, not in cow's colostrum. Colostrum is much richer, but human milk is still four times as concentrated as cow's colostrum.

Fremont-Smith And how about human colostrum as compared with ordinary human milk?

Gyorgy Three times, on the average

Angevine It would be quite possible to examine a large series of udders from cattle, in various stages of disease and milk production One of our graduate students, who was working with *Streptococcus mastitis*, did many biopsies, and then there was another group working on the breast during various stages of pregnancy

Gyorgy. Meconium is an exceedingly rich source of this so-called bifidus factor.

Fremont-Smith: In the newborn?

Gyorgy Yes, which of course is teleologically very exciting and attractive, because of the physiological intestinal habits of breast-fed infants. Meconium is the first medium, so to speak; it is the culture medium which acts as a lure In addition to the meconium, the first bacteria which invade the intestine are *Clostridia*, which appear during the first few days. *Clostridium* contains an enzyme which will break down meconium into an inactive state, but meconium contains an inhibitor which restricts the action of this enzyme. Teleologically, this inhibitor is exceedingly important, because with it the *Clostridium* cannot do anything to the meconium, and the growth-factor remains available for *Lactobacillus bifidus*, which invades the intestine at a later date

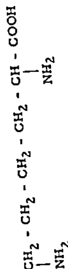
Meyer The meconium contains not a disaccharide, but a polysaccharide

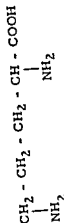
Gyorgy Yes, that is right

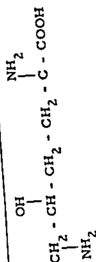
Ragan Dr Sinex, would you care to continue the discussion?

Sinex As you know, collagen contains two unique amino acids, hydroxylysine and hydroxyproline At the present time, we are working on the synthesis of the hydroxylysine moiety of collagen. By feeding rats C^{14}-labeled lysine, we have been able to isolate labeled hydroxylysine. Figure 7 shows some of the relationships that we are thinking about in respect to hydroxylysine and hydroxyproline.

Lysine, proline, and hydroxyproline are constituents of the diet. Lysine







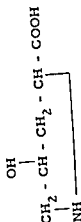


FIGURE 7 The structure of lysine, hydroxylysine, ornithine and hydroxyproline

has been recognized many times in chromatograms of the free amino acids of various tissues While less prominent, proline and hydroxyproline (16) are also present We have found the recently described method of Jepson and Smith (17) useful for detecting hydroxyproline on paper chromatograms of tissue filtrates. Free hydroxylysine has never been observed Gordon (18) and Astrup (19) have found the O-phosphate ester of hydroxylysine in certain tissues of adult and embryonic ox, but the significance of these observations to the question of collagen synthesis is obscure Collagen contains no phosphate.

Mrs Stetten (20) has studied the synthesis of hydroxyproline, using N^{15}-labeled amino acids She first observed that proline is formed from ornithine She then made the rather surprising observation, that while the hydroxyproline of collagen can be formed from dietary proline, labeled hydroxyproline, when fed to rats, did not appear in collagen Dr Stetten felt that her data could be interpreted as indicating that hydroxyproline was formed in the collagen molecule after the peptide bonds were formed In respect to hydroxylysine, we have just completed the first phase of the experiments, and have found that hydroxylysine may be derived from dietary lysine. There is at least a superficial resemblance between hydroxylysine and hydroxyproline, as can be shown by a somewhat distorted structural formula

Of what significance are hydroxylysine and hydroxyproline in connective tissue? We do not know The fact that these amino acids are unique for collagen may mean that their synthesis is an integral part of the synthesis of the collagen molecule, and that the rate of this synthesis may markedly influence that of collagen.

Ragan Dr Krog, will you tell us something about your work?

Krog For the last four years, I have been working in Alaska with the United States Public Health Service, where we have been concerned with the adjustment of man and animals to life in a cold climate. I personally worked on problems relating to the control of body temperature We found that Eskimos have warm feet and hands because they know how to protect themselves by appropriate clothing Many animals, however, we have found have rather cold extremities, some parts of them are at times only 10° C above freezing Artic swimming birds sometimes have a temperature in the webs of their feet which approximates that of the surrounding ice water. This raises the problem of how so-called warm-blooded tissue responds to these low temperatures, As far as I know, no biochemical or histological studies have been made, comparing these tissues with others which we know to have been damaged by prolonged exposure to cold.

Travell. Were these subcutaneous or surface temperatures, that were 10°C. above freezing?

Krog: We measured the temperature in the hoof gland of the caribou, it is a fat gland that lies between the toes This was an attempt to obtain a foot temperature at the most distal point that has some thickness

Travell: How deep was the measurement made?

Krog: About 4 centimeters, so it would be at the central part of the hoof

Fremont-Smith: Is it a lymph gland?

Krog. No; I do not know what the real function of it is It has been suggested that it is a scent gland, whatever that means

Fremont-Smith: Is it vascular? Will it bleed?

Krog: No, not particularly, I should say it is more comparable to the tail fat glands of certain other animals It has a tuft of hair on the surface, around its orifice, and its secreta are quite fatty

Travell: Is it sensitive to the needle?

Krog: No, not very. We used a very fine needle thermocouple which I made especially for this study The thermosensitive junction is lodged within a 25-gauge needle, which still is strong enough to withstand use in the field, and at the same time only causes a minimum amount of trauma For subcutaneous measurements it is always inserted under the skin for a certain distance, so as to reduce influence on the measurements by condition along the needle

Ragan: Dr. Holbrook, will you make a few concluding remarks?

Holbrook: I can only say a word about the work we are continuing to do on the mechanism of the remission in rheumatic disease, more specifically, rheumatoid arthritis We have been intrigued for a long time with the relationship of pregnancy to the remission that occurs in a very high percentage of pregnancies in rheumatoid arthritis We began to study the mechanism of the amino acids in pregnancy, and that led us to the study of the remissions in the disease after such substances as cortisone, ACTH, and so forth, were available We found that the active rheumatoid arthritic has a markedly decreased urinary excretion of certain amino acids, and a moderately lowered blood level, as compared with the normal person, also, that when remission occurs, either by ACTH, cortisone, Compound F, jaundice, or pregnancy, there is a very striking increase in the urinary excretion of several of the amino acids, the most marked of which have been histidine, threonine, lysine, and tyrosine We have been unable to produce this tremendous increase in the urine without a remission occurring. We have tried many nonremission substances, such as high protein diets, amino acids

feeding, testosterone, ascorbic acid, aspirin, epinephrine, and others, without being able to produce this pattern.

Sinex Do you obtain plasma changes with those same amino acids?

Holbrook· Yes, we do The plasma changes are much less marked than those in the urine The urinary output is 100 to 400 per cent increased, whereas the plasma level shows a much smaller per cent rise

Mirsky How soon after the administration of the hormone does the rise appear?

Holbrook It seems to occur about the time when the swelling begins to go away, as near as we can determine clinically, that is, at about the fourth or fifth day This appears to be wholly independent of whether the patient is in a positive or negative nitrogen balance.

Curiously enough, one substance, Compound B, will produce profound metabolic changes without showing the slightest remission factor. The amino acid pattern is very curious in Compound B, in that there is an increase in all the amino acids in the urine, without at all the selective pattern which we see with remission We are now attempting to study the metabolic pathway of these particular amino acids which have seem to be important in the remission situation We know some of their abnormal metabolites, and have methods now for measuring some of them We do not know yet whether this has anything at all to do with the remission factor, except that so far we have been unable to reproduce this pattern without the remission

Sinex The concentrations of the plasma amino acids do not vary a great deal in pathological conditions The regulation compares very favorably with that of blood glucose. A lot can be done here, and it supplies a very fruitful field for investigation.

I am wondering whether you considered nephrosis at all

Holbrook Yes, we have done some treatment of nephrosis, of course, as everybody has, with the steroid compounds and ACTH.

Sinex Do you notice anything unusual about the pattern of the nephrotic plasma?

Holbrook I cannot recall that we did. Do you mean following the treatment with cortisone or ACTH?

Sinex: Just to begin with — taking the nephrotic syndrome.

Holbrook They are all low.

Sinex Did you observe a crisis, to see whether, in that case, they dropped further?

Holbrook· No

Sinex· Were these done by paper chromatography?

Holbrook· No, all of our determinations were done by microbiological methods.

Ragan: I have not called upon the people who are to give their talks in detail later on I should like to say also that I am sorry we shall not have time to discuss the disorders of the connective tissue which are congenitally medicated, such as osteogenesis imperfecta, van der Hoeve's syndrome, and so forth, which might be a rather fruitful field of study for one phase of connective tissue growth I know of one experimenter who is approaching it from a genetic and pathological point of view, particularly with Follis' (21) idea that in osteogenesis imperfecta, the derangement in osteoid may be due to a defect in the osteoblast We are also working, as is Dr Angevine, on lathyrism, but from a somewhat different point of view I agree with what he has said And I do not know whether Dr Holbrook agrees with this, but we have finally become convinced that rheumatoid arthritis is a vascular disease, in its primary lesion.

Holbrook. I would not agree or disagree.

Ragan. Whether the primary lesion is in the blood vessel, or in the connective tissue around the blood vessel, I am not sure We certainly have what I consider to be reasonably sound evidence, that at least the first lesion we see, is in the blood vessel wall

Holbrook I think the clinical evidence for that is also extremely striking We see young women with Raynaud's phenomenon, who begin to have a little swelling of their proximal interphalangeal joints, and their hands turn white and blue Raynaud's phenomenon and rheumatoid arthritis are so closely related that I do not know how to tell them apart with certainty

Fremont-Smith Are you saying the Raynaud's disease is *primarily* a vascular condition?

Holbrook Not Raynaud's disease, but Raynaud's phenomenon I suspect it is a neurogenic disturbance manifested in the vascular system

Fremont-Smith What would you say about rheumatoid arthritis? Do you think it is primarily vascular, or is it possibly neurogenic?

Ragan Suppose that 50 per cent of a given strain of inbred rats will normally develop rheumatoid arthritis Then we frustrate those rats and 100 per cent develop rheumatoid arthritis In that case I think that there is some neurogenic basis for the condition

Holbrook I agree

Ragan But until we obtain that result, we cannot experiment with a human being, it is just impossible

Mirsky I beg to differ with that statement It is not scientific to begin with and it is too dogmatic a statement to make about the question posed What you can or cannot prove in the rat has nothing to do with the question as it pertains to man May I ask you a question

that is more pertinent? What is the vascular lesion that you first observed?

Ragan: It is necrosis of the vessel walls, starting with the intima.

Mirsky. Is there any deposition of any of the polysaccharides?

Ragan: There is very little information on that.

Angevine: Dr. Ragan, Is this a generalized phenomenon? Are those changes also in the synovial membrane?

Ragan: It is the beginning of the nodule.

Angevine. That is what I meant.

Ragan: That is about as far as we can go with the specific lesion in rheumatoid arthritis.

Holbrook: Do you think it is the same as that in lupus?

Ragan: No.

Fremont-Smith: I suppose that one of our difficulties is the search for a single cause. Our friends in the field of physics have taught us a little bit about multiple causality, Gestalt, or pattern, and this seems to me a much more profitable approach. When we are looking for what we call a cause, can we find one of the several dimensions of a problem which are essential to its manifestations? If we can turn off one of those dimensions, Dr. Holbrook, we can stop the disease. For instance, if we roughly divide a disease into various predisposing and precipitating factors, then if we remove these factors we do not contract the disease. Take Raynaud's disease, for example: it must have a predisposing factor which we perhaps do not understand, but precipitating factors might be anxiety, exposure to cold, or the onset of fever. If we eliminate these precipitating causes, then we prevent the manifestation of the disease.

Holbrook: I am interested in that, of course, Dr. Fremont-Smith, but much less so than in a specific mechanism at the site of the lesion. I have the conviction that when disease starts at this site, there must be a specific mechanism which is the same regardless of many so-called precipitating factors.

Fremont-Smith. And that specific mechanism, according to my frame of reference, will be an interaction between a specific predisposing and precipitating cause or causes.

Holbrook. It will be a simple chemical or enzymatic action, probably.

Fremont-Smith: Dr. Holbrook, there is no reason why it has to be simple, but, in any case it will be an interaction. It has to involve all the elements essential to the manifestation, does it not?

Holbrook: Yes, it will be the result of one or forty things.

Mirsky: Perhaps Sir Thomas Lewis (22) put it well, when he

talked about the etiology of disease and stated "When we inquire into the cause of a disease, we are often brought to consider a long chain of relevant circumstances somewhere however, in the chain there is a particular event, which is of cardinal importance to the individual, since it may be said to have set the chain of events in motion in him" It is the "particular event" we usually refer to as the cause, even though it is just one event in a sequence of events The "event" becomes important because of what preceded it.

Holbrook I am thinking of some enzymatic or chemical change, at the cellular level, which produces, shall we say, tissue swelling, or other symptom of rheumatoid arthritis. If we could prevent the mechanism from functioning at that point, then we would have no further anxiety about a sequence of events in that disease, because we would have blocked it at the source

Fremont-Smith But if we block it at any point in the sequence, it is stopped

Holbrook We might have to block twenty channels, perhaps

Fremont-Smith. Yes, if there were twenty channels

Holbrook: Whereas, if we could block it at one point

Fremont-Smith You mean the pathway where the disease is manifested?

Holbrook Yes, that is the place to search

Fremont-Smith I see it now

Mirsky: But even that, I question. Is there such a thing, even on the molecular basis?

Holbrook. I like to think so, anyway

Mirsky Is there a single factor that will cause a clinical syndrome such as, let us say, edema?

Holbrook Certainly

Mirsky I would disagree with that

Fremont-Smith. One of the purposes of the free interchange we have been having is to tap the resources of a group of people in a way that can never take place in the usual scientific meeting, where people get up and make statements at one another I think this has been a very interesting discussion

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THE EXCHANGE OF MATERIALS BETWEEN BLOOD VESSELS AND LYMPH COMPARTMENTS*

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INASMUCH AS the blood capillaries represent an intrinsic constituent of the connective tissues, it is appropriate that consideration be given, on a biological level, to the functional characteristics of the various structural elements, the resultant activity of which makes up the hematoparenchymal barrier. The capillary wall, and its contiguous structures, are interdigitated into a versatile functional entity which enters into a wide spectrum of physiological and pathological processes. We are dealing here with an extremely labile organic unit, the behavior of which is so intimately related to its immediate environment that even minor variations in experimental contingencies have a profound effect on its operation This is a direct reflection of its design, since the terminal vascular bed represents the sensory element of the circulatory system responsible for the delivery to the tissues of nutritive material, in accord with constantly shifting metabolic requirements. Subtle alterations in the hematoparenchymal barrier develop in association with particular fluctuations in metabolic activity. These range from processes involved in its role as a semipermeable membrane in the exchange of fluid and solutes between the blood and tissue compartments, to those concerned with its participation in local defense mechanisms, such as blood clotting, and the infiltration of the tissues with leukocytic elements and antibodies An adequate understanding of the fundamental processes involved in these varied facets of tissue homeostasis, will depend in considerable part upon our ability to relate them to physicochemical changes in particular structural components The bulk of our information in this regard is of necessity purely descriptive, since our methods for the measurement of integrated functional processes on a cellular level provide only relatively crude estimates of such delicate physiological phenomena

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Under normal conditions, the capillary wall acts as a semipermeable membrane, which permits the free passage of water and solutes with a diameter below approximately 100 angstroms, and with almost complete retention of the formed elements of the blood and the plasma proteins (1). However, under abnormal conditions, and occasionally with changes in functional behavior, the perviousness of this barrier is increased to the point where even the cellular constituents of the blood penetrate into the tissue Considerations of blood-tissue interchange have failed to place sufficient emphasis on the multiple nature of the barrier separating the blood stream from the parenchymal cells Actually, the capillary wall proper, i e., the endothelial cells and the cement, represents only a framework onto which are superimposed, on either side, structural elements with a direct influence on the exchange of materials across this structure In passing from the blood stream to the tissue cells, five distinct structural elements are encountered (*a*) a proteinaceous component, presumably from plasma and/or platelets; (*b*) the endothelial cells proper, (*c*) the intercellular cement substance, (*d*) a condensed pericapillary sheath, and (*e*) the connective tissue proper, including the ground substance and its fibrillar constituents (2).

The actual movement of materials across this barrier is governed primarily by physicochemical forces, including osmotic and hydrostatic pressures, with the net exchange being a function of such factors as the size of the pores of the separate structures, the relative thickness of each structural component, the diffusion characteristics of the different molecules, and the lipid solubility of the materials involved. The capillary surface across which the exchange occurs, consists of an extensive cellular area with a small intercellular zone (less than one per cent) There is some disagreement as to whether the bulk of the exchange occurs across the entire capillary surface (3), or is restricted to the more pervious intercellular cement (4) The evidence is conclusive that the penetration of large molecular aggregates and formed elements occurs between the cells through the intercellular cement

Pappenheimer (5), in recent studies with labeled isotopes, suggests that even molecules as small as water probably diffuse in and out of the capillary through an extremely small portion of the total available surface The functional characteristics of the intercellular cement have been found to be influenced by many factors of both local and systemic origin In essence, the basic structure involved is a porous cement substance, presumably a calcium proteinate. Superimposed on this, either by electrical, chemical, or surface tension forces, is a large molecular component which plugs most of the large pores in the cement, con-

siderably reducing the over-all perviousness of the structure It is suggested that the pore size, the relative number of pores, and their shape, are determined both by the nature of the cement, and its absorbed component. Both of these constituents have a variable relationship in different tissues, and under different environmental conditions.

Meyer: What evidence is there that this is a coated and fibrous meshwork?

Zweifach I do not wish to give the impression that the cement is known to consist of an interlacing network of fibers onto which is coated a proteinaceous element. There is no objective evidence to support this concept However, the experimental data clearly indicate that the barrier acts as a membranous structure in which the pore size, shape, and so forth, are determining factors. Inasmuch as permeability characteristics of the cellular constituents cannot be related to this type of structural organization, one must assume that the remaining structural element, the endothelial cement, represents the major physical barrier Evidence in support of a plasma constituent, in addition to the cement, which influences the perviousness of the barrier, will be presented later I must emphasize that most of the evidence is indirect in nature, and that the concept has arisen from microscopic observations on the vascular bed of living animals.

Fremont-Smith Are there any electron-microscope studies of capillary walls?

Zweifach Yes; they indicate the presence of a porous cement substance. However, in preparing such material for section, one invariably removes an important constituent of the cement, the material which lines the pores, and so to speak waterproofs the structure.

Fremont-Smith Does the intercellular space show any fibrous material, and is there histological evidence for it?

Zweifach I do not believe that a fibrous structure of sufficiently small pore dimensions has been demonstrated by electron microscopy

Meyer· I am not sure whether the structure is based on observation or is postulated A layer, or layers, of protein absorbed to the capillary wall, would probably explain your observations if the protein molecules were not tightly packed, and without assuming a fibrous network of a proteinaceous packing material

Zweifach We have no direct evidence concerning the submicroscopic picture of the interendothelial cement. Observations concerning its porosity and the changes in perviousness which develop under different conditions, are deduced from particular changes in the behavior of the vessel in the living animal. The extent to which the porosity

of the structure can be influenced by absorption of selective proteinaceous material from the blood, represents an intriguing mechanism for regulating the passage of material Several investigators, including Danielli (6), have postulated a mechanical factor, with the blood platelets plugging the pores However, whether the secondary layer represents a component of the blood-clotting system, as believed by different investigators, or is a combination of the cement with blood platelet elements, remains speculative (7) The increased capillary permeability which develops in conditions involving blood platelet depletion or defects in the blood clotting system, is suggestive of a direct relationship between these two processes and the perviousness of the capillary wall (8) The term "inert" may be applied to the cement substance to indicate that it is not to be considered an intrinsic portion of the living endothelium, but rather as an extraneous material coating the outer surface of the cells I do not mean to indicate that the cement substance is chemically inert, or that it has a fixed physicochemical structure The circumference of the capillary wall consists of from two to four endothelial cells, joined together as an elastic tube. The cement substance would appear to be continuously secreted by the activity of viable endothelium. Factors which act on the cement substance cause the endothelial cells to withdraw from one another In experiments where the blood capillary is exposed to a weak solution of silver nitrate, introduced with a micropipette, the cement material is precipitated, apparently in the form of a silver proteinate When carefully done, this staining may be achieved without interrupting the blood flow through the capillary vessel The blackened cement is then gradually washed away by the flow of blood through the vessel Since this occurs without affecting the integrity of the vessel wall, it is reasonable to assume that the intercellular material is being replaced In instances where the [illegible]

ensues rapidly (9)

In actively growing tissues, such as may be observed in transparent chambers inserted into the rabbit ear, or in granulation tissue, the intercellular cement appears soft and quite adhesive to particulate matter In normal tissues, the capillary wall is elastic, and can be stretched to a considerable degree between two microneedles In contrast, similar manipulation of the vessel wall in granulation tissue results in a ready rupture and separation of the endothelial cells The capillary wall of the newly formed vessels is highly permeable, even to plasma proteins It is interesting to note that as the physical characteristics of the vessel

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In actively growing tissues, such as may be observed in transparent chambers inserted into the rabbit ear, or in granulation tissue, the inter cellular cement appears soft and quite adhesive to particulate matter In normal tissues, the capillary wall is elastic, and can be stretched to a considerable degree between two microneedles In contrast, similar manipulation of the vessel wall in granulation tissue results in a ready rupture and separation of the endothelial cells The capillary wall of he newly formed vessels is highly permeable, even to plasma proteins t is interesting to note that as the physical characteristics of the vessel

wall undergo change, i e, the cement becomes less adhesive, the surrounding ground substance becomes gelated, and the perivascular sheath becomes more firm, the high permeability of the vessels to large molecular aggregates disappears.

The nature of the cement has been found to be affected by changes in electrolyte concentration The presence of calcium is essential for the maintenance of the normal characteristics of this material. In experimental conditions where the calcium content of the circulation is artificially reduced, the cement goes into solution and is washed away (9). In tissue culture preparations, the growth of endothelium in the form of sheets or tubes is likewise dependent on the presence of adequate amounts of calcium. For example, if the calcium content is reduced, the endothelial sheath begins to fall apart, cells tend to move away from one another and to round up With the reintroduction of calcium, these cells again flatten out and join to form a continuous sheath or tube of endothelium (10). Apparently a small amount of calcium (about ten per cent of normal) is sufficient to maintain some adhesiveness In experiments with calcium-deficient media, the endothelial cells can be readily separated from one another by microneedles This is in contrast with experiments using normal concentrations of calcium, where it is almost impossible to separate two contiguous endothelial cells with microneedles without destroying the cells It is of interest that the substitution of magnesium or strontium is not an adequate replacement for calcium with regard to this function.

To the best of our knowledge, cells are kept in an organized contiguous relationship with their neighboring units by virtue of the presence of an extraneous coat. This was demonstrated many years ago by Hober (11), and others, and shown to be related to the electrolyte make-up of the surrounding environment, particularly calcium No other known forces exist which can maintain the structural form of units such as the capillary Experiments by Chambers and Cameron (10), dealing with mesonephros tubules in tissue culture of chick kidney, emphasize the importance of calcium and pH in maintaining the integrity of cellular membranes of this type

Ragan Could we go back to the term "inert"?

Zweifach I agree that it is a poor adjective to use to describe the cement Actually, the cement substance represents a labile structure, with a variable physicochemical make-up, which may be in a continuous state of change. The presence of a relative calcium deficiency is sufficient to change the characteristics of the cement In calcium-free media, the absence of an adhesive cement substance does not necessarily indicate that the material is not being produced, it is probable that it is being

secreted in a soluble state. On the basis of the shape and tone of the endothelial cells, and their reactions to histochemical staining, we assume that the viability of the capillary endothelium is not a factor under these conditions. The viability of the endothelial cells is also attested to by the observation that the cells reconstitute their normal contiguous relationship when calcium is restored to the perfusion medium It is highly significant that a structural element, which constitutes only about 0.2 per cent of the entire capillary surface, represents the major factor affecting the bulk of exchange between the blood and the tissues

Meyer. This 0 2 per cent is based on perfusion studies?

Zweifach Pappenheimer (5), on the basis of hind limb perfusion studies, concluded that the exchange of water and lipid insoluble materials must occur across an extremely small fraction of the total capillary surface On the other hand, oxygen, carbon dioxide, and lipid soluble materials probably permeate through the cell surface (12), thereby having a much larger area of exchange.

In this regard, consideration should be given to the work of Flexner and co-workers (3) These investigators believe that statistical analysis of their data, concerning the passage of labeled isotopes, indicates that the exchange of materials occurs across the entire capillary surface However, there is a good deal of disagreement among biomathematicians as to the interpretations of some of the assumptions which have been made.

Fremont-Smith What you are saying, Dr Zweifach, is that your histochemical picture, with these points of contact between cells, would correspond roughly with the kind of picture which Pappenheimer provides mathematically?

Zweifach Our own conclusions, arrived at from the point of view of a cellular physiologist, coincide fairly well with the evidence provided by the perfusion technique of Pappenheimer

Fremont-Smith The bulk of water passes through the intercellular cement region, does it not?

Zweifach Yes, the bulk of fluid exchange, which includes water and dissolved electrolytes, probably occurs there

Gaudino Dr Zweifach, it has been postulated that proteins normally pass in appreciable amounts through the capillary wall Do you agree with this assumption?

Zweifach On the basis of the studies dealing with the passage of labeled albumin from the blood stream into the lymphatics, a slow exchange is indicated (13) However, regional differences in the extravascular loss of blood proteins exist. If we accept the pore type of

structure as the primary barrier regulating the passage of material, consideration must be given to the fact that this is not a uniform feature The pore population is a heterogeneous one, with pores ranging in diameter from several angstrom units to a small percentage which are as large as several hundred angstrom units. These differ not only in size, but also in shape. Evidence exists that the relative distribution of pores may vary in different capillary beds, depending on the local factors which influence the cement. An important limiting condition in this regard may be the nature of the material which is absorbed onto the inner surface of the cement. This gives rise in different areas to capillaries with a relatively large number of pores of sufficient size to permit the passage of protein.

As the capillary tree progresses towards its venous end, the number of large pores would appear to be progressively greater than exists on the arterial end. Here again the gradient relationship is not fixed, but varies with the makup of the circulation. Thus, the extent of the outward passage of proteins from the circulation will depend not only upon size, shape, and charge of the molecule, but upon factors influencing the porosity of the capillary barrier.

Gaudino Dr. Zweifach, you suggest that the capillary membrane — if we call both the cells and the intercellular substance the membrane — has the property of changing its physical and chemical characteristics, depending on physiological circumstances However, this postulate seems to modify the basic premise of Starling's (14) hypothesis, which is that the capillary membrane behaves as a semipermeable inert membrane, and that the movement of fluids and solutes is exclusively dependent on the magnitude of the forces acting on both sides of it and not on the membrane itself If, according to your view, the behavior of the membrane is quite variable, then the basic classical picture should be seriously reviewed My point is this if one postulates the existence of a semipermeable membrane, one is by definition assuming that the membrane *per se* will not change. If the membrane is susceptible to change, then the postulated picture should be different.

Zweifach The Starling hypothesis implied the bulk movement of fluid and dissolved constituents through the capillary wall, with elements retained above a certain molecular dimension The direction of movement was postulated to depend upon the interplay of the hydrostatic against osmotic forces More recently, it has been shown that diffusion represents an important factor in blood-tissue exchange. The average pore size of the capillary wall is calculated to be between 30 and 45 angstrom units (15) This permits the free diffusion of water and most electrolytes, depending upon the concentration gradient and

osmotic forces Exchange of materials occurs much more rapidly by diffusion processes than by hydrodynamic forces The net exchange between the blood stream and the tissues is influenced by a variety of factors, Pappenheimer and others have amply discussed the extrinsic variables However, insufficient emphasis has been placed on the dynamic character of the capillary barrier itself, not only in relation to regional differences, but also in a given set of vessels under varying experimental conditions Changes in the pore size, in a critical range, may influence the relative importance of hydrostatic and osmotic forces as opposed to diffusion forces

Fremont-Smith Dr Zweifach, I have been led to believe that hydrostatic and osmotic forces operated in accelerating or decelerating diffusion, and that a distinction could not be made between diffusion and the role of these forces I assume that osmotic pressure results from an interference with diffusion by the solute, and hydrostatic pressure from an increase in the diffusion process by virtue of pressure, so that placing these in different categories would be at least open to question

Zweifach I believe the consensus of opinion indicates that outward filtration and inward diffusion can occur independently This was demonstrated in perfusion experiments, where the effective capillary pressure was raised without influencing the diffusion of freely exchangable substances

Fremont-Smith You are now speaking of infiltration pressure of water as against diffusion of other substances That was not what I meant I was talking about diffusion or filtration of the same substance I think when you shift to a different substance, it introduces another variable

Zweifach Our own attempts at studying capillary permeability have been comparatively crude when compared with the technique employed in isolated limb perfusion studies However, one must take into account the effects introduced by removal of a structure from the body and its perfusion with artificial media on the permeability characteristics of the structure Certainly, the multiplicity of control mechanisms which are constantly operating to maintain the capillary barrier in accord with the requirements of local tissue metabolism are completely disrupted in this type of preparation Factors concerned with the precise functional characteristics of the hematoparenchymal barrier are of necessity overlooked These are of special importance with respect to regulatory processes leading to abnormal or disease states

Ragan If a capillary at one site may have a different permeability from a capillary at another, it seems to me, we must take into consideration the milieu in which the capillary rests, and say that that affects

diffusion and filtration as well We can observe the varying effects of hormones in different tissues of the body The baboon sex skin, for example, is a peculiar tissue because of the medium it is in; the cell at that particular place responds entirely differently from the way it does at another site

Mirsky. Or the reabsorption of water by a distal tubule

Ragan Yes; I do not think one can say the capillary in a cat's hind limb will be the same all through the limb. It seems to me that there are a great number of variables in the cat's hind limb that will be averaging out

Meyer The difficulty, it seems to me, is in part caused by the fact that the concepts about pore size, dialysis, and filtration, are based on the structure of membranes composed of homogeneous material, while the living membranes have an extremely complex organization I wonder how many layers are interposed between the capillary and the lymph vessel, and how thick this is in terms of angstrom units.

Zweifach· The distance between separate capillaries varies from 50μ in tissues such as skeletal muscle, to 100μ or more in tissues such as the skin. Considerable variability is encountered, both with respect to the total number of capillaries and the distance across which material must diffuse in and out of these vessels. Structural differences in different regions of the body impose a complex set of considerations The relative importance of factor *A*, the internal lining; factor *B*, the endothelial cell, factor *C*, the perivascular sheath, factor *D*, the cement, and factor *E*, the connective tissue ground substance, must be evaluated for each separate tissue Each of these may represent a limiting factor, not only with respect to its own properties, but also with reference to its effect on the other constituents of the barrier.

Fremont-Smith. At one time, I made a study of anterior horn cells in the spinal cord, in thick serial sections In every one of the forty or fifty that I studied, at some place a capillary wall was adjacent to the anterior horn cell, so that the pericellular and the pericapsular space were confluent, and there was practically no distance at all between them The capillary wall lay almost against the anterior horn cell There would not have been room there for more than one to three red cells, which would perhaps provide an example of the simplest relationship between a cell to be fed, and a capillary wall.

Zweifach In certain areas, an epithelial membrane is superimposed outside the capillary proper. This is encountered in the eye, the brain and the glomeruli of the kidney In these situations a specialized type of permeability exists, usually involving some form of secretory activity. The establishment of the separate contribution of the different

structural elements is of primary importance in the etiology of different pathological states Each of these constituents can undergo alteration, independently, of an active change in other constituents of the vessel barrier I shall present some photographic illustrations of changes which develop in the capillary wall under different experimental conditions The interendothelial cement may be visualized by introducing a fine suspension of graphite into the blood stream. The carbon particles adhere only to the intercellular cement, and progressively outline the cell borders (Figure 8) The remainder of the capillary wall is unstained, so that one obtains preparations in which only the cell out-

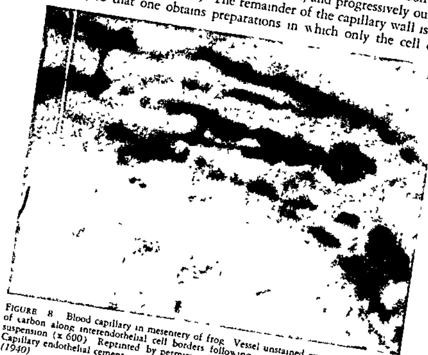


FIGURE 8 Blood capillary in mesentery of frog Vessel unstained except for deposition of carbon along interendothelial cell borders following intravenous injection of carbon suspension (x 600) Reprinted by permission, from Chambers, R and Zwiefach, B W Capillary endothelial cement in relation to permeability *J Cell & Comp Physiol* 15, (1940)

lines are visible Factors, such as calcium deficiency and excess, changes in pH, through their effect on the cement material, will influence the character of the carbon lines in the cement material. Excess calcium causes increased accumulation of carbon, not only along the cell borders but also over the entire surface of the cell. Deficient calcium, or acid pH, results in the progressive washing away of the adherent carbon from the intercellular cement. The loss of cement is accompanied by a progressive edematous condition in the tissues

Mirsky: Does that mean, therefore, that this represents a beginning disintegration of the cell?

Zweifach: The appearance of carbon particles along the cell borders is a slow phenomenon in normal vessels. Under abnormal conditions the deposition of carbon is considerably enhanced. In addition, injury to the cell results in an over-all sticking of carbon to the endothelial surface Under such conditions, the endothelial cell may be undergoing degeneration In calcium-free perfusion experiments, with the washing away of the cement material, the endothelial cells pull away from one another and begin to round up. However, this condition is reversible, and a more normal appearance can be restored by the addition of calcium to the perfusing solution This would appear to indicate that the endothelial cells had not been irreversibly damaged.

To what extent can we carry the analogy between the extraneous coat of unicellular organisms, and that of the endothelial cell? Unicellular forms secrete a cement material over their entire surface. What then is the reason for the absence of a cement substance on the inner surface of the capillary? The possibility exists that the cement is rapidly washed away as soon as it is formed. As previously indicated, only under abnormal conditions is evidence obtained for the development of adhesive layer over the entire surface of the endothelial cell (Figure 9).

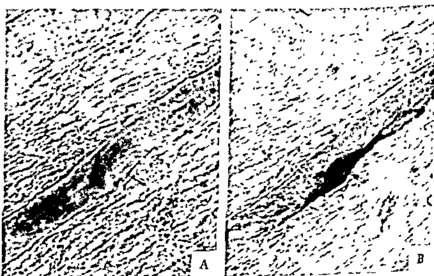


FIGURE 9 Responses of blood capillary to microtrauma (*A*) Tip of microneedle broken off and left in place to right of vessel Carbon injected intravenously Blood flow from left to right (*B*) Five minutes later, note carbon accumulation, chiefly below point of injury over surface of endothelial cells Rat mesentery (x 100).

Changes in the distribution of adhesive material on the capillary wall may also be introduced by micromanipulative methods through the injection of calcium salts or acid materials Local changes in pH bring about a breakdown of the cement material, and an increased permeability of the vessel wall. In experiments where the intercellular material is demarcated with carbon, perfusion with a calcium-free medium, or an acidified mixture, considerably accelerates the dissipation of the carbon.

It has been possible, by means of a micropipette, to introduce silver nitrate locally, and to bring about a blackening of the intercellular cement substance This can be done without disrupting the capillary circulation With time, the cement is washed away. The replacement of the stained cement by new material occurs without disrupting the flow through the vessel

Freemont-Smith And that has not destroyed the endothelial cell itself?

Zweifach· Excessive concentrations of silver nitrate will irreversibly damage the endothelial cell Under these conditions, no regeneration of the cement material is observed Hence the endothelial cells serve an important function in the continuous replacement of intercellular material The capacity of the endothelium to reconstitute the intercellular substance has been found to be affected in conditions of inflammation and other forms of injury (16)

The tone or elasticity of the capillary wall is a reflection of the tone of the endothelial cell proper. This property of the cell is to a considerable extent a factor of the water content Under shifting conditions of tonicity, the endothelial cells can become either swollen, or shrink to the point of retraction from one another. These changes may be demonstrated by micromanipulative means Thus, the injection of hypotonic solutions into the tissue adjacent to the capillary causes them to swell, often to the point of obliterating the internal lumen of the vessel. The micro-injection of hypertonic solutions actually causes the endothelial cells to shrink away from one another (Figure 10) This will result in the extravasation of red blood cells through the newly formed spaces between the cells

Travell How long does it take the endothelial cells to recover from that kind of treatment?

Zweifach The changes in cell shape produced by tonicity phenomena with a micropipette, are transient in character The cells resume their normal appearance within a few minutes

Travell: What tonicity would that be?

Zweifach Usually increasing the tonicity of the physiological saline

by about 30 to 40 per cent suffices to produce irreversible changes in endothelial tone It is probable that changes of this magnitude are rarely encountered in physiological situations



FIGURE 10 Application of 1 5 per cent NaCl adjacent to upper surface of capillary results in formation of opening in which a red blood cell is trapped (at pinched left side of cell) Rat mesentery (x 400)

Fremont-Smith. Dr Zweifach, is it possible to obtain enough tonicity to cause a cell to do what that cell seems to be doing, that is, to open up and let red cells out, and then for that same cell to recover within a few minutes and behave perfectly normal again?

Zweifach. The transient character of the change in the perviousness of the capillary wall is demonstrated in experiments where Evans' Blue (T-1824) is injected intravenously The linkage of this dye with the blood albumin permits one to evaluate the outward passage of such material. Under normal conditions the T-1824 is confined to the capillary, as visualized in the mesentery With the local injection of hypotonic saline into the tissue, a tinge of blue color develops in the immediate area. The outward passage of colored albumin persists only for a period of from 15 to 20 seconds, following which the wall reconstitutes itself. Thus, in addition to changes in the nature of the cement material binding the endothelial cells, environmental conditions which affect cell

tone and shape may serve to bring about the outward passage of the normally retained blood constituents

Conditions which give rise to abnormal changes in the intercellular cement also affect local homeostasis It should be emphasized that changes in the cement occasioned by electrolyte disturbances, or by alterations in pH *per se*, were not always accompanied by the adhesion of platelets and leukocytes Similarly, changes in the endothelial cell, which involve only the factor of tonicity, are rarely accompanied by thrombus phenomena Microtrauma, or the administration of histamine locally, are invariably accompanied by the deposition of platelets along the inner capillary surface

Ragan. When you did the experiment with silver nitrate in which the cement seemed to disappear, did a normal state return in a matter of minutes?

Zweifach· The return to normal conditions following silver nitrate occurred over a period of from 60 to 90 minutes

Gyorgy Is there a stimulus in scorbutic animals, and does one see any pathology?

Zweifach Changes in the capillary wall develop in scorbutic animals, observations have been made chiefly in the mesentery of the guinea pig (17) The appearance of the capillary bed during the period of progressive depletion is superficially normal The adrenal glands of these animals already show a complete depletion of ascorbic acid at this stage However, it is during the latter stages of vitamin C deficiency that lesions appear in the capillary bed, chiefly on the venous side Numerous petechial hemorrhages develop in association with progressive weakening of the capillary wall, as evidenced by handling with microneedles The normal appearance of the capillary wall in scorbutic animals is comparable to the situation in adrenalectomized rats maintained on sodium chloride In both instances the apparently normal appearance can be readily disrupted by the introduction of even minor stress Two important deficiencies appear to develop in the capillary barrier during the progression of the scorbutic state Initially, there is a weakening of the perivascular sheath, probably accounting for the increased fragility of these vessels Later, the interendothelial cement becomes deficient This deficiency is especially apparent in the venous capillaries, where changes of as little as $+2°C$ in the temperature of the irrigation fluid result in extensive extravasation of red blood cells

Gyorgy That is very interesting, and fits into the physical picture

Fremont-Smith I was in Dr Zweifach's laboratory in 1939, and saw these things under the microscope and there was no question about what happened when perfusion with calcium-free material was carried

out. One could see the stickiness, and the cells adhering and then coming away again as soon as normal plasma was returned Although this material has been published, it is still quite new to most of us in terms of our functional thinking about capillary processes. Is that a fair statement to make?

Zweifach Previous studies have tended to emphasize the extreme thinness of the endothelial cell as an important factor in the exchange of material between blood and tissues. However, this fact by itself cannot account for the type of permeability exhibited by the vessel wall Histochemical studies have indicated that the endothelial cell possesses no unusual characteristics Our recent studies with tetrazolium salts have indicated that the endothelial cells are metabolically active, the relative order of their activity being comparable to that of vascular smooth muscle The precise level of metabolic activity apparently is related to the capillary blood flow. For example, frequently two adjacent capillary vessels are encountered in mesenteric tissues, where only one contains an active circulation of blood The endothelium of the capillary with an active blood flow becomes stained with tetrazolium, whereas the other vessel remains uncolored.

In order for the endothelial cell to participate directly in the exchange of water and other electrolytes, highly specialized metabolic processes must be present, involving considerable expenditure of energy No evidence for this type of metabolism exists. We were therefore forced in our thinking to fall back upon the intercellular concept Thus far, no objective evidence has been presented to contradict *this hypothesis* Actually, n [illegible] objective support for the per [illegible] s- cussion is designed [illegible] n its broader implications, so as to shed light on the separate contribu*tion of the different structural elements involved*

Fremont-Smith Hemodynamic exchange between cells through cement substance, and gas exchange and some lipid-soluble material exchange through the cells; is that right?

Zweifach: Yes.

Sinex: How do you think lipoproteins leave the plasma?

Zweifach We have no direct evidence in this regard. Molecular complexes of the size of lipoproteins probably cross the vessel wall to a very limited degree, with their permeation depending upon their molecular size and configuration.

Sinex: I *think your point* about abnormalities in protein metabolism affecting the permeability of the capillary bed is very useful In the nephrotic syndrome, one finds low plasma proteins, and edema, and

yet this edema is difficult to correlate with the absolute level of the plasma proteins, and explain on that basis. At the same time one has this edema, there is a tendency for the lipoproteins of the plasma to accumulate. This additional concept of a proteinaceous coating of the exchange areas between the capillary cells, may lead to a better understanding of why the plasma of nephrotics accumulates lipoprotein, and the nature of nephrotic edema.

Gaudino: This concept of a capillary membrane with variable permeability properties seems to contradict the basic assumption of an essentially inert membrane postulated in Pappenheimer's (15) theoretical treatment of this problem. However, it is surprising that Pappenheimer arrives at the same conclusion as Dr Zweifach, that the passage across the capillary wall for most lipid-insoluble substances is effected through the spaces between the endothelial cells

Mirsky. Hasn't Dr. Zweifach indicated that his use of the word "inert" is not the way it was just used

Gaudino My point is this. if the basic assumption of an inert capillary membrane is proven not to be so, then the whole theoretical treatment has to be fundamentally modified.

Mirsky: The data presented thus far indicate that it is far from inert.

Zweifach. The perfusion studies on the excised hind limb preparations involve, for the most part, the circulation through skeletal muscle, Under the conditions of the experiment, the integrity of the capillary barrier is maintained by an artificial perfusate containing defibrinated blood and red blood cells The precise magnitude of the changes encountered in an *in vitro* system, under an arbitrary set of control conditions in relation to those operating in the capillary bed *in vivo* remains to be demonstrated. Since the perfused system is kept in a static state, except for experimentally induced physical variables, the physiological lability of the vascular bed is completely eliminated

Mirsky Would you postulate that this applies to the so-called blood-brain barrier?

Zweifach The blood-brain barrier requires special consideration, since it consists, in addition to the capillary endothelium, of a layer of epithelial cells closely applied to the small blood vessels This is reflected in the highly specialized type of permeability which is encountered in the brain vessels, especially in relation to lipid-soluble materials

One aspect of capillary structure which has been almost completely neglected, is the presence on the inner surface of the endothelial wall of a thin layer closely adherent to the endothelium and the cement. This structure is so thin as to be invisible through the microscope in all but exceptional circumstances The lining material would appear to

represent some constituent of the blood which is absorbed onto these surfaces. The nature of the constituent involved has not been established The intimate relation of the inner vessel surface to local thrombus formation, suggests that the blood proteins concerned with the clotting mechanism may be related to this feature. Perfusion experiments, in which the blood proteins were replaced by various synthetic or naturally occurring colloidal agents, indicate that the permeability of the wall may be altered in this manner, irrespective of the precise osmotic properties of these materials. A possible explanation is the substitution of different colloidal substances as the material waterproofing the interstices or pores of the capillary membranes, thereby affecting its permeability characteristics Such effects have been demonstrated, not only for proteins such as albumin, but also for substances such as gelatin and PVP (polyvinylpyrrolidone) In experiments where the capillary system was perfused with colloid-free mixtures, the naturally occurring lining of the vessel could be washed out, and the progressive edema which followed could then be studied (18) The addition of various substances to the perfusion medium was then carried out in an attempt to restore normal permeability characteristics Crystalloidal solutions were completely ineffective Small amounts of colloid produced a restorative effect on permeability disproportionate to their osmotic pressure values

The addition of blood platelets to the perfusion fluid likewise served to reconstitute permeability of the capillary wall. The platelet factor would appear to operate by virtue of a true plugging of capillary pores The mechanism, however, is not a simple one, since the platelet action is dependent on the presence of other serum proteins, and apparently upon the precise physical characteristics of the vessel surface This phenomenon appears to be regulated, not so much by the total number of platelets, as by a critical physicochemical relationship between these elements and the vessel wall.

Other investigators have shown, that apparently it is possible to displace the normally present lining in the capillary wall by the administration of materials with different surface-active properties Among the agents believed to exert their effect by this particular mechanism, are basic proteins such as clupeine (19). The displacement of the protein, which normally lines the vessel by other agents, results in an altered permeability of the capillary. Experiments with heparin, to be discussed later, also point to a comparable mode of action with reference to an increased capillary permeability.

The inner capillary lining represents a labile entity which can be altered in various functional disturbances, as well as in pathological

conditions It is probable that many aspects of altered permeability, previously attributed to changes in colloid osmotic pressure with protein depletion, may be ascribed to alterations in the protein layer absorbed to the inner surface of the cement barrier

There are no visible accumulations of platelets on the capillary wall under normal conditions However, with extremely fine microtrauma, platelet adhesion to the inner endothelial cement becomes apparent This has also been demonstrated by Samuels and Webster (20), who stained the intercellular materials and showed the platelets lined up on the interendothelial borders The reaction then spread progressively onto the entire surface of the endothelial cell proper.

Fremont-Smith But even in the most normal capillary bed there are some platelets at the pores at the intercellular junctions, is that right?

Zweifach Several investigators have presented evidence to indicate that various states of increased capillary perviousness may be favorably altered by the intravenous injection of platelet materials (6, 8) The platelet suspension serves to counteract the altered permeability of the wall The assumption therefore has been made that a certain percentage of the circulating platelets remain adherent to the vessel wall under normal conditions However, visible accumulation of platelets appears only concomitant with the development of an increased adhesiveness of the vessel wall

Under normal conditions of the circulation, the leukocytes roll along the periphery of the stream, propelled by the movement of the blood These cells become adhesive only when in contact with an injured vessel wall An increased adhesive state may also be induced by perfusion with media containing excess calcium The normal red cell represents an extremely plastic structure which does not display adhesive properties similar to those of the white cells Erythrocytes are passively trapped in endothelial openings, or become enmeshed in thrombi, and show a tendency to rouleau formation under conditions of reduced blood flow This type of cell adhesion represents a temporary physical attraction which is easily disrupted by mechanical forces

A prominent feature of the capillary wall is the presence of a sheath of delicate fibrils imbedded in a membranous structure closely applied to the outer endothelial surface The sheath varies in consistency and thickness in different regions of the vascular bed Being a condensation of the connective tissue ground substance, it presents a definite barrier to the passage of substances into and from the capillary lumen It is interesting to note that when leukocytes pass through the capillary wall, they at first come to rest in the space between the endothelium and the pericapillary sheath Red blood cells also usually enter the

tissue spaces under conditions which weaken this supporting structure. In Figure 11, a photograph of a small venule, the sheath surrounding the vessel is clearly visible. Its presence about the true capillary is more difficult to demonstrate in the living state because of the extremely thin nature of the membrane.

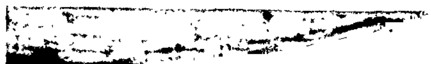


FIGURE 11 Extravasation of red blood cells and carbon particles through pericapillary sheath pricked with microneedle Frog mesentery (x 600)

Meyer: How is that made visible here? It is not stained, is it?

Zweifach: In Figure 11, which shows the living capillary on the venous side of the bed in the rat mesentery, no staining procedure was used The supporting nature of the pericapillary sheath is demonstrated by micromanipulation experiments. When the sheath is torn with the tip of a fine microneedle, the endothelium herniates out at that point and frequently shows spontaneous rupture (Figure 12).

It is my impression that the more common forms of increased capillary fragility are a consequence of a disturbance or weakening of the pericapillary layer. The question arises whether such a membraneous sheath presents a significant hindrance to the passage of water and small molecules. An interesting comparison with reference to the permeability characteristics of such sheaths is found in the perineurium of nerves,

such as the sciatic nerve. Such sheaths have been found to limit the passage of ions to and from the nerves proper, as evidenced by the effect of their removal on the action potentials of these structures. The analogy

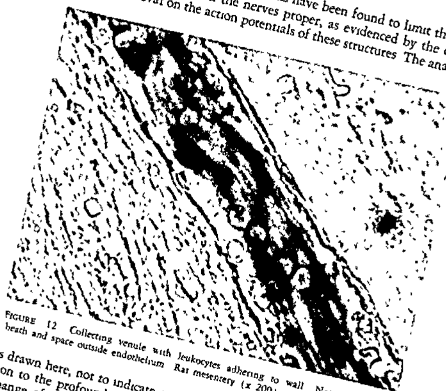


FIGURE 12. Collecting venule with leukocytes adhering to wall. Note perivascular sheath and space outside endothelium. Rat mesentery (x 200)

is drawn here, not to indicate an identical situation, but to bring attention to the profound effect which such barriers may exert on the exchange of small molecular materials.

Evidence for the development of important changes in the pericapillary sheath has been obtained in different experimental conditions. For example, in scorbutic guinea pigs the sheath is easily ruptured by minor micromanipulation, leading to massive extravasation of material from the blood into the tissues. The repair of such microtrauma is limited, and frequently is not apparent during the several hours of observation, in contrast to the fairly rapid repair in normal animals. When testicular extracts with hyaluronidase activity are introduced with a micropipette onto the vessel wall, the pericapillary sheath becomes eroded at this point, and the vessel frequently develops a petechial hemorrhage in this region (21). It is interesting to note that the testicular extract has no visible effect on interendothelial cement. This supports the contention

that the cement and the ground substance are two distinct chemica entities Both the thickness of the sheath and its precise perviousnes would appear to represent important factors limiting the exchange o both cellular and dissolved elements from the blood stream into th connective tissue proper.

Gaudino. Did you inject the hyaluronidase systematically?

Zweifach: In the experiments cited, the hyaluronidase extract wa introduced directly into the tissues immediately adjacent to the capillar wall in the mesentery of the anesthetized rat. The final structura component involved in blood-tissue exchange is the connective tissu proper. This structure presumably has no major influence on diffusio processes The injection of diffusible dyes into the ground substanc of the connective tissue results in a rapid, even diffusion, similar to that observed when such dyes are introduced into a block of gelatin Attention should be given to a number of features which could limi the free movement of water and dissolved substances in this medium The term "tissue fluid" actually is a misnomer, since the ground substance exists as a gel When one introduces particulate matter, such as carbon, directly into the ground substance with a micropipette, the carbon remains highly localized as a small bleb It does not spread unless the pressure used to introduce the material is elevated to excessively high values The gelated ground substance can be transformed into a fluid by a number of environmental factors, including hyaluronidase extract Furthermore, the avidity of the various fibrous elements, and the ground substance for cations, may likewise change, and in turn alter the avidity of these structures for water. Ascorbic acid deficiencies appear to give rise to abnormalities in the ground substance, which in turn leads to increased capillary fragility and edema

An attempt was made to provide information concerning the biochemistry of the capillary endothelium as a basis for studies dealing with abnormal states, such as inflammation, by using different histochemical methods For the past several years we have employed the

(22) The tetrazolium compound is unusual, existing in its oxid state as a colorless, water-soluble salt Its oxidation-reduction potential is in a range which permits it to act in living systems as a hydrogen acceptor, and be reduced to a colored end product The colored formazan is insoluble and becomes deposited within the cytoplasm The "staining" obtained in this manner differs from conventional staining, in that it reflects an active metabolic process. Cells which are damaged or killed,

or whose metabolism is altered by various metabolic poisons, do not show any reduction of tetrazolium

The method permits two types of analysis The tissues can be sectioned and examined histologically for the extent and pattern of tetrazolium deposition This approach has proved exceptionally useful in the study of complex structural units, such as the kidney The reductase activity of the separate constituents of the nephron can be identified A second application of the method involves the extraction of the reduced tetrazolium, and its measurement with a suitable photocolorimeter. The combined qualitative and quantitative approach was found to be valuable in studies dealing with the effects of various metabolic inhibitors The application of the technique to the study of the terminal vascular bed, presented several unique problems Quantitation was not possible, since the blood capillaries could not be isolated for such measurements However, quantitative studies on extremely small arteries and veins are possible In the initial studies, it was found that the capillary endothelium and vascular smooth muscle did not react with tetrazolium as did conventional tissues. Several modifications were introduced, including a longer period of incubation, and the addition of a variety of substrates It was found that incubation of mesenteric tissues in a medium containing tetrazolium and succinate, or mannose, provided a uniform coloration of the endothelium and smooth muscle * The reduction of tetrazolium by vascular endothelium, provides for the first time a means of estimating changes in endothelial metabolism

Mirsky I do not quite understand how you introduce your substrates Would there be a piece of mesentery lying in a suspended position in a medium?

Zweifach Tetrazolium agents may be introduced in several ways Originally, homogenates of different tissues were prepared and incubated *in vitro* Later, as an extension of this work, the tetrazolium reductase activity of intact tissues was studied by incubating tissue slices *in vitro* as for microrespiration experiments (23) In the present studies, because of the extreme thinness of mesenteric structures, it was possible to incubate excised mesentery directly in a suitable tetrazolium mixture Several other techniques have been employed The tetrazolium can be injected intravenously or subcutaneously in low doses over a period of time Unfortunately, some of the nerve centers in the brain become stained with the material to such a degree that convulsions and death ensue Antopol (24) has found that neotetrazolium, administered in chronic doses, will stain spinal ganglia, and various collections of nuclei, in a brain stem proper.

*Fried G H, and Rosenberg, E. K Unpublished data

Another possible experimental procedure was the perfusion of tetrazolium solutions through isolated organs This has been done successfully with kidney, liver, and the skeletal muscle of hind limb preparations The studies reported here involve the *in vitro* staining of either mesenteric sheets removed from the animal in the living state, or of fresh frozen section of different organs

In excised spreads of mesentery, capillary endothelium, arterial smooth muscle, lymphatic endothelium and smooth muscle, as well delicate fibers, stain following incubation with neotetrazolium The possible applications of this method are obvious. We have added a variety of substrates, as well as specific metabolic inhibitors, designed to interfere with cell metabolism in a predictable manner. It is hoped that such an approach will provide information concerning endothelial and smooth muscle changes, prior to the appearance of visible pathology in these structures.

In Figure 13, the delicate nature of the staining is evident The cytoplasm of the endothelium contains a punctate deposition of tetrazolium, while the nucleus remains clear. In addition to the staining of smooth muscle, many of the connective tissue cells actively reduce tetrazolium Under certain conditions, the mast cells lying alongside of the capillary vessels show an unusual capacity to reduce tetrazolium.

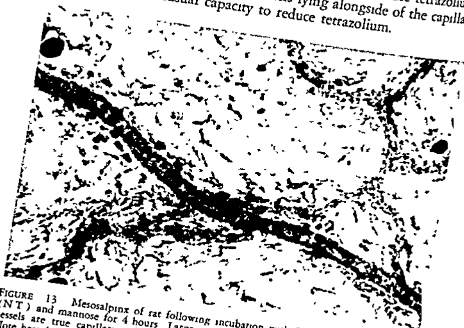


FIGURE 13 Mesosalpinx of rat following incubation with 0 5 per cent neotetrazolium (N T) and mannose for 4 hours Large vessel crossing field is terminal arteriole Finer vessels are true capillaries Endothelium and smooth muscle colored by reduced N T Note heavily colored mast cells along arteriole (x 150).

Fremont-Smith The cytoplasm is stained and the nucleus is clea but in the brain it is the nucleus that is stained, is that right?

Zweifach The term "nucleus" used in the discussion of the central nervous system refers to a collection of cells in that area The staining of these nerve cells involves the cytoplasm We have never observed active reduction of tetrazolium by any of the nuclei in the various tissues which have been studied

A word of caution should be interjected at this point it concerns the implication that tetrazolium reduction may be attributed to a specific enzyme system It is obvious that tetrazolium can act as a hydrogen acceptor at a number of different points in the metabolic cycle The use of specific substrates, such as sodium succinate, may exaggerate the dehydrogenase activity of this particular step in the metabolic cycle However, changes in the reduction of tetrazolium, even in the presence of succinate, involves a series of steps prior to succinic hydrogenase, as well as subsequent to its participation in the cycle In other words, the tetrazolium method provides an over-all measure of total reductase activity, and it is possible only in special cases to determine just which metabolic enzymes are involved in the experimental conditions under study

The application of the tetrazolium technique to the secretory activity of the adrenal cortex, illustrates the possibilities which this approach may afford It has been possible to stain selectively different zones of the adrenal cortex under specific experimental conditions When the same gland was studied with conventional histological or histochemical techniques, no distinction in zonal activity was possible One striking example was observed in experiments where the activity of the outer zona glomerulosa was suppressed by the administration of DCA A sharp differentiation in terms of the capacity to reduce tetrazolium was found between directly contiguous cells of the glomerulosa and adjacent fasciculata

A somewhat analogous situation was encountered in the capillary bed Those capillaries, which had an active flow of blood through them, reduced tetrazolium Adjacent capillaries, which were devoid of blood flow, did not stain These experiments were done on excised tissues However, before the tissue was removed the circulation was observed through the microscope, and a map drawn of the circulatory pattern in the different vessels It was thus possible to correlate the extent of staining with the distribution of blood flow

Mirsky But the stain has to get in through the exterior of the vessel

Zweifach Since the studies were performed on excised tissues, the

tetrazolium should penetrate equally to all of the vessels in the tissues Hence the staining, or lack of vascular staining, must be referable to some other factor Various connective tissue elements, including macrophages, show an active reduction of tetrazolium. This becomes exaggerated in inflamed areas Studies of the inflammatory reaction with this technique would appear to provide a useful approach to this problem

In frozen sections of different parenchymal tissues, the walls of the arteries and veins are well stained with tetrazolium. It is of interest that the smooth muscle coat of the veins does not reduce the tetrazolium as actively as that of the small arteries and arterioles Connective tissue fibers or sheets appear as unstained clear zones.

The lymphatic vessels in the mesorchium or mesovarium of the rat are well stained by tetrazolium In the living state, the lymphatic capillaries are difficult to visualize (Figure 14). However, in most tissues they form a rather extensive network, and constitute an important constituent involved in tissue homeostasis The lymphatic capillaries are more permeable than the corresponding blood capillaries For example, when particulate matter is injected into the connective tissue, it will gradually appear in the lymphatics, but never in the blood capillaries When a suspension of carbon is injected into the tissues un-



FIGURE 14 Network of lymphatic capillaries in mesorchium of rat, indicating difficulty of clearly visualizing these vessels in living state Blood capillaries are dark vessels in upper right corner of figure (x 80)

der pressure, the material frequently moves directly into the lymphatic capillaries The injection of colored dye into the connective tissue spaces is followed by its rapid appearance in the lymphatics

Apparently the cement material binding the lymphatic endothelium together is much weaker than that present in the blood capillaries When one teases the lymphatics with microneedles, the endothelial cells separate quite readily In several experiments in the rat, carbon was injected with a micropipette into a terminal lymphatic in a retrograde direction The carbon does not penetrate the lymphatic into the tissues, but adheres to the intercellular material of the endothelial wall When one injects testicular extracts into the connective tissue together with carbon particles, slight pressure on the tissue is sufficient to force the carbon into the lumen of the lymphatic

Apparently blood proteins do not return to the blood stream by way of the capillaries, but do so by way of the lymphatics Evidence in the literature indicates that labeled protein does not reappear in the blood circulation after injection into the tissue locally (25) Introduction of albumin, colored with T-1824, by means of a micropipette into the connective tissue, is followed by the progressive appearance of the blue material in the terminal lymphatics Presumably this represents the diffusion of albumin into the lymphatics and not the dissociated dye, since an intense coloration of the lymphatic lumen develops within several minutes

Ragan Do these lymphatics also have perivascular sheaths?

Zweifach The larger lymphatics (at least 30 to 50μ) have a clearly visible outer sheath However, the terminal extensions of the lymphatic capillaries appear to blend with the connective tissue proper This makes it difficult to delineate sharply the lymphatic wall

The retrograde injection of thorotrast, or India ink, into the terminal lymphatics, indicates that there is a physical barrier between the lymphatic lumen and the connective tissue proper However, the injection of dyes, such as water blue, which are normally retained by the capillary wall, usually pass directly from the lymphatic into the connective tissue

Evidently the lymphatic barrier is highly permeable in comparison to the capillary wall The terminal lymphatics stain with tetrazolium The endothelium closely resembles that of the blood capillary An interesting observation is the presence of a thin, clear space between the lymphatic endothelium and the connective tissue sheath

Fremont-Smith Do you know what those spaces are?

Zweifach They may be observed along lymphatic vessels until they interconnect to form structures larger than 50μ in diameter The

Zweifach The evidence presented in this dicussion emphasizes the importance of extracellular elements in the exchange of materials between blood and tissues. In previous discussions, the importance of the extreme thinness of the endothelial cell has been emphasized, and it has been suggested *that it is the factor which permits the cell to act as* a highly permeable porous barrier. However, this criterion is fallacious There are innumerable examples of thin layers of protoplasm which maintain a highly selective type of permeability. Evidence concerning the metabolism of the endothelial cell indicates that it duplicates all of the metabolic phenomena of other living cells. Until evidence is provided to the contrary, we must assume that the permeability characteristics of the endothelial cell are identical with those of other cellular constituents This necessitates some explanation other than that of cellular permeability as the primary consideration in capillary permeability.

The exchange of material between blood and tissues, especially under abnormal conditions, has been found to be related to the local release of chemical agents Two substances which have been implicated in these reactions are heparin and histamine. Heparin has been shown to be prominent in certain tissues, such as the liver and in the mast cells adjoining the blood capillaries throughout the body, and its release would appear to involve the discharge or dissolution of heparin-containing granules from cells of the mast cell variety This can occur with unusual rapidity under conditions of simple stress Histamine, on the other *hand, is believed to be released following the development* of proteolytic activity (28) The separate effects of these substances on the several constituents of the capillary wall have been studied under different experimental conditions.

Heparin has a considerable modifying effect on the reaction of the capillary and the circulating blood cells to local trauma. As previously described, the sticking of leukocytes to the vessel occurs almost exclusively in the venous capillaries. Following heparinization, the leukocytes in the circulation become rounded, and are carried along by the blood without appreciable adhesion; they do not change their shape when in contact with the vessel wall, nor do they remain stationary at any point This is in contrast to the control animal, where mild rubbing of the capillary wall with a microneedle is sufficient to introduce localized adhesion of blood leukocytes to the affected area

Heparin almost completely abolishes the leukocytic reaction to microtrauma. A sticking of leukocytes could be induced only by unusually severe trauma, sufficient to tear the tissue or to destroy the endothelial cells A tear in the vessel wall continued to bleed without evidence of the formation of a thrombus for periods up to two to three

minutes This is unusual, since in normal tissues even extensive disruption of the endothelial wall is closed off by a plug of leukocytes and platelets within 20 to 30 seconds. Of particular interest is the absence of an adhesive coating on the surface of the leukocytes in heparinized animals, as demonstrated by the failure of carbon particles to deposit on the surface of these cells.

Microtrauma is associated with the massive elaboration into the blood stream of a gelatinous exudate, or precipitate, in which the blood cells become enmeshed Previous heparinization interfered with the appearance of this material, but could not suppress its formation in instances where cell trauma was severe. In no case did heparinization subsequent to trauma result in the disappearance of the material

Ragan Did the white cells stick?

Zweifach Heparin was effective in disrupting the usual sequence of platelet and leukocyte adhesion, following local injury, when introduced directly into the tissue with a micropipette Extensive local heparinization resulted in a weakening of the capillary wall, and considerable extravasation of red blood cells Under these conditions, active diapedesis of leukocytes was inhibited

Following local tissue injury and the appearance of a gelatinous mass of cells, the repair process in normal animals was characterized by a slow washing away and breaking off of the intravascular masses Whether this material is identical with the sludge referred to by Knisely (29) is not clear

A gelatinous precipitate appears as a consequence of chemical and mechanical tissue trauma, and not as a consequence of reduced blood flow or stasis (Figure 16) There is not sufficient evidence to indicate whether this substance is elaborated by the cell, or appears in response to substances liberated from the cell which interact with the plasma

When vessels, which have been injured this way, are perfused with artificial solutions, it is possible to wash away this accumulation of material most effectively with calcium-free media In this respect, the masses resemble the cement type of material which appears on the cell surface in response to tissue injury

Mirsky Is this digested by testicular extracts?

Zweifach No

Ragan This must be some substance that moves toward the capillary wall from the injured tissue

Zweifach This type of injury reaction appears in the capillary, even when the trauma is introduced some distance away from the vessel wall There is definite evidence to indicate that the reaction is the conseque

Zweifach The evidence presented in this dicussion emphasizes the importance of extracellular elements in the exchange of materials between blood and tissues. In previous discussions, the importance of the extreme thinness of the endothelial cell has been emphasized, and it has been suggested that it is the factor which permits the cell to act as a highly permeable porous barrier. However, this criterion is fallacious. There are innumerable examples of thin layers of protoplasm which maintain a highly selective type of permeability. Evidence concerning the metabolism of the endothelial cell indicates that it duplicates all of the metabolic phenomena of other living cells. Until evidence is provided to the contrary, we must assume that the permeability characteristics of the endothelial cell are identical with those of other cellular constituents This necessitates some explanation other than that of cellular permeability as the primary consideration in capillary permeability

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sufficient to produce vasodilation, is without visible effect either on the permeability of the capillary wall or on the behavior of the formed elements of the blood However, with higher concentrations of histamine, a pronounced effect on the capillary wall *per se* can be observed.

The micro-injection of as little as 01 γ of histamine, produces a pronounced vasodilation in the capillary bed The injection of concentrations of 0 1 γ, or higher, results in capillary damage The total volume injected is a drop ranging from 2 to 4 μ in diameter (Figure 17).

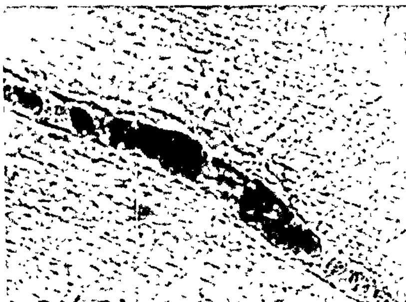


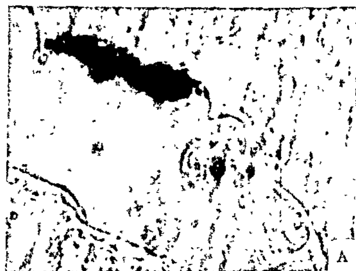
FIGURE 17 Production of capillary damage and stasis by histamine A drop of histamine (0 1 per cent) introduced with micropipette below capillary where dark spot persists Blood cells move toward injured area from both directions, indicating increased out ward loss of fluid from vessel Rat mesentery (x 100)

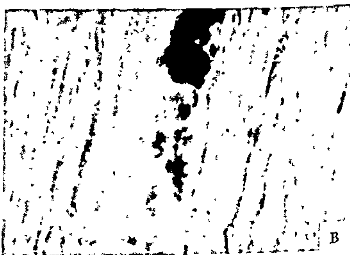
The toxic effects of histamine are twofold First, the endothelial cells begin to swell, and the endothelial nucleus protrudes into the lumen of the vessel Coincident with this, a weakening of the intercellular cement becomes obvious, and numerous red cells penetrate into the tissues Changes in the adhesive properties of the vessel wall, or of the leukocytes, do not develop until the histamine produces extensive cellular damage in the area adjacent to the vessels under observation.

Smex That is far too low a concentration to study the tetrazolium system with histamine as a substrate, is it not?

of the diffusion of chemical principles from the injured site to the vessel.

Histamine has also been implicated in a variety of vascular reactions. Its physiological significance in terms of a specific action has not been demonstrated. The local injection of concentrations of histamine, just





following
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igh lighted
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vironmental conditions. Following the adhesion of leukocytes to the injured vessel wall, certain of these elements begin to emigrate through

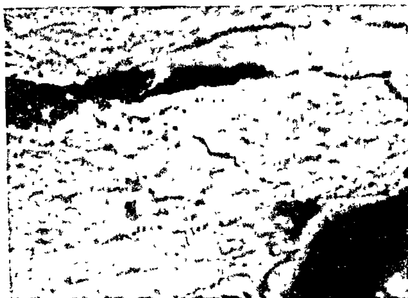


FIGURE 18 Gradual washing away of carbon adherent to injured capillary in upper part of field Blood flow from right to left Carbon deposited temporarily distal to injured site Adjacent capillary unaffected Feeding arteriole (dark vessel) at lower right. Rat mesentery (x 400)

into the perivascular space This would appear to represent an active movement on the part of the leukocytes

Fremont-Smith. Is the active movement encouraged by pressure from within?

Zweifach The application of pressure with a microneedle outside the capillary, and against the outward movement of the leukocyte, stops the progression of this process However, it does not actually reverse it

Fremont-Smith But it is really ameboid?

Zweifach The outward migration of leukocytes can occur in vessels in which the flow has been interrupted by temporary occlusion with a microneedle Frequently as many as three to four leukocytes will find their way through the vessel at the same point in the wall

Figure 19 demonstrates that an integral part of the leukocytic reaction is the development of surface adhesiveness It shows a leukocyte in the blood stream into which carbon has been introduced, and the

Zweifach: Studies on the effect of histamine on tetrazolium reactions of endothelium are contemplated, but have not as yet been carried out

The extravasation of red blood cells through the capillary wall is a passive phenomenon, involving the passage of the cell through the cement substance by virtue of the pressure exerted by the blood flow The red cell does not appear to adhere to the vessel wall prior to its extrusion Micromanipulation studies have brought to light several interesting features concerning the structural make-up of the red blood cell. It is a highly plastic unit which appears to represent a loose envelope, surrounding a fluid interior When the red blood cell in the capillary is pricked with the tip of a needle, it disappears, leaving almost no ghost or remnant behind However, with trauma or excessive handling the cell becomes gelated It can now be stretched between two microneedles and cut in two. In this state the cell acquires adhesive characteristics, and will stick to the vessel wall, or to blood leukocytes.

Fremont-Smith. Can a jellied red cell recover?

Zweifach: The evidence in this regard indicates that a gelation phenomena is an irreversible reaction. However, whether varying degrees of these reactions can develop and still be reversible remains to be demonstrated

The micromanipulative technique permits the introduction of graded degrees of trauma, and thereby enables us to separate the various sequelae of tissue damage which develop with unusual rapidity under normal circumstances. Studies indicate that an increase in the perviousness of the capillary wall is accompanied by changes in the cement substance prior to the accumulation of blood cells, or platelets to form thrombi Likewise, extravasation of red blood cells and leukocytes may be induced, and the sequence of these reactions studied The evidence clearly indicates that cellular migration, as well as that of particulate matter, occurs between the cells and not through the endothelial cell, as has been postulated

Some consideration should be given here to the question of the phagocytic properties of vascular endothelium As previously mentioned, some degree of selective accumulation of particulate matter is exhibited by the capillary wall as a consequence of changes in the intercellular cement. In addition, with tissue injury the inner surface of the endothelial cell bcomes adhesive (Figure 18). However, there is no evidence that this represents true phagocytic activity, since the particulate matter does not appear outside the vessel, except where a definite weakening in the wall is demonstrable The carbon particles, which have accumulated along the inner surface of the damaged endothelial cell, are progressively lost into the blood stream with a restoration of normal en-

ironmental conditions. Following the adhesion of leukocytes to the injured vessel wall, certain of these elements begin to emigrate through

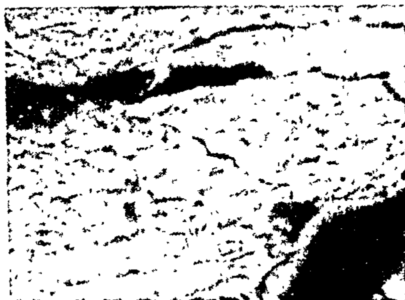


FIGURE 18 Gradual washing away of carbon adherent to injured capillary in upper part of field Blood flow from right to left Carbon deposited *temporarily* distal to injured site Adjacent capillary unaffected Feeding arteriole (dark vessel) at lower right Rat mesentery (x 400)

... perivascular space This would appear to represent an active ... ocytes

... nent encouraged by pressure from within?

Zweifach The application of pressure with a microneedle outside the capillary, and against the outward movement of the leukocyte, stops the progression of this process However, it does not actually reverse it

Fremont-Smith But it is really ameboid?

Zweifach The outward migration of leukocytes can occur in vessels ... has been interrupted by temporary occlusion with ... leukocytes will find ... the wall

Figure 19 demonstrates ... of the leukocytic re-action is the development of surface adhesiveness It shows a leukocyte in the blood stream into which carbon has been introduced, and th

surface of the cell is seen to be studded with fine particles of carbon The progressive increase in leukocyte adhesiveness on the venous side

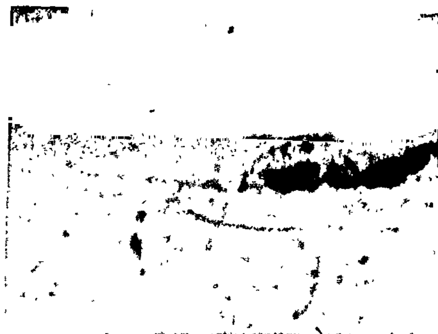


FIGURE 19 Leukocyte adhering to wall of venous capillary Dark area across photo indicates blood flow containing carbon suspension Surface of leukocyte studded with carbon particles Rat mesentery (x 800)

of the capillary bed would appear to indicate that some factor associated *with the venous character of the blood has an influence on this property.* The mechanical reversal of blood flow in the capillary bed, forcing venous blood into normally arterial channels brings about an increased *sticking of carbon and leukocytes in these vessels*

An interesting effect of heparin is the augmented permeability of the capillaries in animals which have received sufficient quantities of this agent to render the blood incoagulable. The capillaries become unusually susceptible to even minor changes in temperature, or handling with microneedles Evidently heparin in some way interferes *with* a factor instrumental in maintaining the normal state of the capillary wall My impression is that this indicates some constitutent of the blood *clotting mechanism is normally associated with the capillary* wall, and that heparin alters this relationship.

The administration of from 1 to 5 mg of cortisone to rats has a definite influence on the terminal vascular bed, within as short a period

as from 30 to 45 minutes. The circulating leukocytes become rounded spheres, and do not adhere on the venous side of the bed, as in control preparations The response to local trauma is modified, since leukocyte accumulation and diapedesis are almost completely absent The reactive hyperemia following local trauma or the local introduction of histamine, is also considerably blunted and rapidly reversed by both topical and systemic cortisone.

There is a good deal of evidence in the literature relating cortisone to vascular reactivity (30). Hence many of the changes in blood flow, subsequent to the administration of cortisone, may be ascribed to its effect on the vasoconstrictor tone of the affected blood vessels.

Gaudino. Did cortisone produce any changes in the case of local trauma?

Zweifach. The reaction of endothelium to direct injury, involving the accumulation of extensive masses of gelatinous material at the site of injury, was not affected by cortisone pretreatment The toxic effects of histamine, with reference to endothelial cell damage, were likewise not altered by cortisone There was no enhancement of the repair process in locally traumatized vessels. The injection of cortisone systemically or locally did not prevent the development of an increased outward loss of T-1824 from the blood capillary, following local microtrauma.

Meyer Did you do any experiments with ethylenediaminetetraacetic acid?

Zweifach No, we have not carried out experiments of this type.

Meyer I wonder whether or not the cement substance itself has the properties of a complex acid, so that if we change the pH we suppress the dissociation, and it is in competition for example, with heparin

Zweifach· Our own experiments, in a very general way, indicate that heparin, both locally and systemically, can change the permeability characteristics of the capillary wall Its specific mode of action remains of course speculative. Whether it acts by producing a clumping of platelets, or by affecting the vessel wall directly, has not been demonstrated

Gaudino Have you studied the effect of the water-soluble compound, desoxycorticosterone glucoside, on leukocytes, as you have cortisone?

Zweifach We have not carried out studies with DCA comparable to those with cortisone, or cortical extracts Cortisone was applied topically in known concentrations by dissolving the crystalline material in blood serum, and then diluting the sample accordingly

Angeime I should like to make one comment in relation to the role of capillaries in these processes in various sites in the body in

tumors, and so forth. I think that Dr. Zweifach's hypotheses explain many things we have observed experimentally. However, it is difficult for me to conceive of the platelets filling these openings all the time, perhaps because I usually associate platelet thrombi with injury. When we look at capillaries, we can observe platelets quite readily, but we do not see them routinely. Your answer might be, "Well, we do not look for them," but it is one thing that bothers me.

Zweifach Experiments implicating a platelet factor in the increased capillary permeability following total body x-irradiation, have been presented by Cronkite and associates (8) They observed a considerable increase in the red blood content of the thoracic lymph after radiation. This could be circumvented by pretreatment with platelet suspensions

Fremont-Smith: Does this occur rapidly?

Zweifach In perfusion experiments, the addition of platelets causes a decrease in capillary permeability back to normal within a period of several minutes

Ragan. In thrombocytopenic purpura, there is an increased permeability of the capillary wall for red cells. When these patients are given ACTH or cortisone, the permeability decreases, but the platelet count does not, uniformly

Zweifach: Are you referring to the formation of capillary petechiae?

Ragan Yes, but it may also occur with suction, if a Dalldorf pump is used.

Zweifach: We have encountered an increased capillary fragility, chiefly as a consequence of changes in the perivascular sheath. However, it is obvious that alterations in the blood-clotting mechanism, involving the blood platelets, may also be present

The phenomenon does not involve a reduction *per se* in the total number of circulating platelets This can be demonstrated by injecting gum acacia, which rapidly depletes the active circulation of blood platelets without evidence of a change in capillary permeability. The platelet-capillary wall relationship may involve some changes in the normal mechanism assocated with the combination of platelet material and cement.

Sinex. I should like to comment on the action of tetrazolium on the endothelial cell. I do not know much about tetrazolium However, I do know that when oxygen consumption is followed manometrically in

ing some particular characteristic of endothelial cells.

Zweifach. The fact that glucose or mannose serve to bring about a reduction of tetrazolium by capillary endothelium and smooth muscle, does not of course indicate that the glucose enters directly into the reaction Some general manifestations of cell metabolism may have been altered, making it possible for the cell to use tetrazolium as a hydrogen acceptor.

Mirsky. Of course, it is not necessarily correlated with oxygen consumption

Zweifach Although in general the over-all tetrazolium reductase capacity shows a trend comparable to the relative oxygen consumption of the cell, changes in either process can occur independently of one another.

Mirsky What does insulin do in this case?

Zweifach We have not studied the effects of insulin

Mirsky Due to the fact that there is more and more evidence that insulin influences the transport of glucose and other hexoses across the membrane into the cell, I wonder what insulin would do in combination with the tetrazolium.

Zweifach The use of tetrazolium as an indicator of specific facets of cell metabolism should be interpreted with extreme caution, since the compound can act as a hydrogen acceptor at many levels of cell metabolism

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to diffuse into the totality of its space of distribution, provides a measure of the water volume sought The calculation is the simple division of the total amount introduced by the concentration, measured after complete and uniform distribution The main criterion of exclusive distribution into the compartment investigated is extremely important for the accuracy of the determination This is particularly pertinent in the case of substances used for the estimation of extracellular volume, since many have been found to enter some part of the cellular compartment, rendering such measurements invalid

Other important requirements for substances used as indicators are the following They should be evenly distributed throughout the compartment, if this uniformity of distribution does not occur, such a substance would not serve its purpose even if the first condition were fulfilled They should not be formed or destroyed in the body, except if these processes can be accurately quantitated, and they should not cause a redistribution of body water, either by their specific properties or by the amounts introduced They should be slowly eliminated, nontoxic, and of easy chemical determination

The general technique for the use of such substances involves a single intravenous injection of a measured amount, and after sufficient time has elapsed for uniform distribution, a blood sample is taken for the determination of the concentration of the indicator at equilibrium The main assumption implicit in this procedure is that the concentration in the plasma or blood is a fair sample of equilibrium concentration throughout the space Immediately after injection, plasma [illegible]

concentration is rapidly achieved and plasma level becomes constant By dividing the amount injected by plasma concentration at this moment, the volume of distribution may be determined, and will be equal to the volume of the compartment being measured if ideal conditions have been met Ideal conditions required for an indicator of total body water volume are almost completely satisfied by two of the substances currently used for that purpose heavy water (D_2O) and tritium oxide However, no such complete fulfillment of requirements is met by any of the substances so far used for extracellular volume measurements

Returning now to body water subdivisions, extracellular water has been subdivided into two other compartments intravascular and interstitial The intravascular compartment comprises water contained inside the vessels, and the interstitial compartment contains the water located between the vascular system and the cells By water contained [illegible]

INTERSTITIAL WATER AND CONNECTIVE TISSUES

MARIO GAUDINO

Department of Surgery
New York University College of Medicine

THE WORK I shall present was done on the macroscopic level, in contrast to the microscopic level on which Dr Zweifach's work was based It deals with the total water of the body, and its subdivisions in different compartments We have studied the changes observed in these compartments, both in volume and in chemical composition.

First, I shall review the methods we used, because this will give us a clearer understanding of the problem and the difficulties involved, both in the measurements and in the interpretations.

Water is the main chemical component of the body, comprising about 70 per cent of its lean mass. It is generally subdivided into two main portions· water contained inside the cells, and that which is outside the cells The importance of these two main subdivisions of body water lies in the fact that although these water spaces have very different chemical and physiological characteristics, they are able to coexist and interchange materials, seemingly because they are separated by membranes which appear to have the role of maintaining their individuality The study of changes in the volume and composition of these two fluids, both in physiological and pathological conditions, constitutes at present one of the main problems in understanding the basic functions and dysfunctions of the body.

Until recently, procedures available for the measurement of these different water spaces have not permitted simple and quantitative analyses of their alterations However, new and improved methods have been devised which have simplified these determinations and increased their accuracy The usual technique has been to measure, simultaneously and independently, total body water and extracellular water, and calculate intracellular water by simple difference

In general, the measurement of any water compartment in the body is commonly accomplished by using an indicator, usually extraneous to the organism, which has to satisfy the essential condition of uniform and exclusive distribution throughout the space to be measured Any substance with this property, when introduced into the body and allowed

as occurs in gastric and intestinal mucosa, salivary glands, red blood cells, connective tissue and testes (4, 5). It is also known that animals deprived for a long time of potassium in their growing period, or in their diets, replace their tissue potassium with sodium (6). However, in spite of these shortcomings, total body chloride or sodium, combined with their concentration in the serum, was the only method available for the measurement of the so-called extracellular space and its changes (4)

Since this procedure demanded the sacrifice of the animal and was inapplicable to man, another approach was employed for the determination of changes of extracellular volume Assuming a certain initial value for extracellular space, chloride and sodium balances were performed, and the data obtained utilized to calculate these variations (7)

At present, total exchangeable chloride or sodium mass in the body can be measured by means of radioactive chloride or radioactive sodium. Under the assumption that such total exchangeable ion is in solution, and that plasma concentration is a fair representative of that solution, both chloride and sodium spaces have been determined by means of these radioactive indicators (8, 9, 10, 11, 12, 13) Spaces thus measured have the same shortcomings as those derived from data on stable sodium or chloride

Chloride space is smaller than sodium space, and therefore has been considered as a more valid approximation of extracellular space, especially since sodium has been found to exist in muscle, and in the whole body, in a higher proportion than chloride (4) From this last observation it was concluded that a certain proportion of sodium in muscle was intracellular

Bromide, both radioactive and stable, has been described as a good indicator of extracellular fluid (12, 13, 14, 15) The basic claim for its use as such has been that its distribution in the body is exactly the same as chloride The ratio of chloride/bromide in tissues is almost exactly one, and consequently both seem to have identical distribution (14, 15) Obviously the use of bromide has the same objections as the use of chloride

One of the most widely used substances for measuring extracellular volume has been sodium thiocyanate. It had the great advantages of rapid equilibration, extremely slow excretion, and of being very easily measured chemically However, since the first description of its use by Crandall and Anderson (16), it was known that thiocyanate entered certain cells (erythrocytes, those of the gastric mucosa, and so forth) Therefore, the volume of distribution of this indicator includes a certain variable portion of the cellular space It was therefore sug-

the vascular tree we refer, of course, to plasma water Water co inside the red blood cells is intracellular water

Plasma comprises about 4.5 per cent of the total weight of t (2) This intravascular water compartment has also been m by the dilution method described previously. The indicator most used for this purpose has been Evans' Blue (T-1824) which itself specifically to the albumin molecules of the plasma and fore a specific indicator of albumin volume of distribution Ot stances utilized have been albumin tagged with radioacti (I^{131}), hemoglobin, or antigens. The values for plasma vol tained by utilizing all these substances simultaneously, agree w error of the methods. If correction is made for the water co plasma, the measurement of the volume of distribution of any indicators will permit the calculation of plasma water volum volume of both extracellular and intravascular water are kn terstitial water mass may be easily calculated by differenc determinations, coupled with total body water measurement a relatively simple method for the study of changes occurring ferent body water compartments. The crucial requirement for determinations is to obtain an accurate estimation of extracellul volume

Extracellular space has been measured by different procedur first attempt was made histologically in muscle, by visual exam of frozen preparations in which the proportion of spaces betw cells was compared to that of the total mass of tissue From 1 17 5 per cent of the total muscle mass was thus found to be int or intercellular space The fact that the total amount of chloride ured chemically in muscle, if assumed to be dissolved in tissue wa the same concentration as that of a plasma ultrafiltrate, yields a vo of solution identical to the interfibrillar volume estimated visually to the conclusion that chloride was of exclusive extracellular distr tion, and therefore a good indicator for extracellular volume meas ments (3). Sodium, coupled with chloride, and on the basis of similar distribution, was considered under the same assumption as good approximation for extracellular volume estimations (4).

However, there are many indications that these two ions are no exclusively extracellular, and are accumulated in certain tissues in highe amounts than extracellular distribution would warrant. In many tissue the sodium/chloride ratio is higher than the corresponding ratio in an ultrafiltrate of plasma Such tissues are striated muscle, bone and cartilage. On the other hand, there are other tissues in which the sodium/chloride ratio is lower than the one of an ultrafiltrate of plasma

its molecular weight, and partly to the fact that inulin undergoes hydrolysis when heated (27). Our present concept is that each sample of inulin is composed of a number of variably sized molecules which give an average molecular weight This weight will vary with the source of the carbohydrate, and the procedure utilized for its extraction

Inulin does not enter the red blood cell, is not concentrated by the liver, and is considered not to traverse the renal tubule This latter property is the basis for its use as a measure of glomerular filtration rate It has the further advantages of being physiologically inert and of exerting negligible osmotic effect, a circumstance which will reduce the possibility of drawing water from the cells No enzyme of animal origin is known to hydrolyze this carbohydrate, and when given intravenously it is completely recovered in the urine

However, inulin, like sucrose and mannitol, has the disadvantage of being rapidly excreted by glomerular filtration After a single intra-

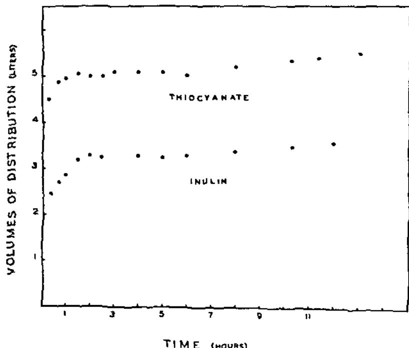


FIGURE 20 Simultaneous comparison of the volumes of distribution of inulin and thiocyanate in function of time after single intravenous injection in a nephrectomized dog Reprinted, by permission, from Gaudino, M, and Levitt, M F Inulin space as a measure of extracellular fluid *Am J Physiol* 157 387 (1949)

gested for use only as an index of changes, and not for estimation of the absolute value of extracellular space. In pathological conditions, and in febrile states, it has been observed that thiocyanate space increases so markedly as to become almost equal to total body water (17). Under these conditions, therefore, it must either enter the cells, accumulate somewhere in the body, or both. circumstances which would invalidate its use as a measure of extracellular space, or as an indicator of its changes. It has also been shown that thiocyanate becomes bound to protein to a variable degree, and that this binding, if not reckoned with, will introduce an important error in the determination (18)

The order of magnitude of the spaces of distribution measured with all these substances may be judged by simultaneous determinations of thiocyanate, Na^{24}, Cl^{38} spaces in the dog Reported data of such an experiment have given spaces of distribution of 35 6, 27 6 and 24 7 per cent of the body weight respectively (11) Spaces of distribution of Cl^{36}, Br^{82}, and Na^{24} in dogs, give a range of 25 to 26 per cent of the body weight for chloride, 27 per cent for bromide, and 29 per cent for sodium (12) Under the main assumption of extracellular location of these ions, chloride seems to be a better measure of extracellular fluid volume, simply because of its smaller space of distribution In the search for a better indicator of extracellular space, and to avoid the possible entrance of electrolytes into cells, several carbohydrates to which the cell membrane was known to be impermeable have been studied. These were mannitol (19, 20), sucrose (21, 22), and inulin (23, 24).

They present the problem of a very rapid renal excretion by glomerular filtration, and in the case of mannitol, of being metabolized It has been reported that sucrose in dogs is incompletely recovered in the urine, however, in man complete urinary recoveries are obtained It has also been determined that inulin is not metabolized, because complete urinary recoveries have been obtained in all species studied (25)

Inulin has many advantages over any of the other substances used to measure extracellular space. As it is not an electrolyte, and is lipoid-insoluble, this reduces the possibilities of its traversing cell membranes either by ion exchange or by lipoid solubility Its large molecular weight renders less likely its passage through membrane pores However, its actual molecular weight is still not agreed upon. Chemical tables, depending on the number of fructose molecules believed to compose its molecule (either 3 or 6) list its molecular weight as either 504 or 990 9 Other data in the literature indicate an order of magnitude of 5,000, or equivalent, to 30 fructose molecules (26). This discrepancy is due partly to the different methods utilized in the determination of

its molecular weight, and partly to the fact that inulin undergoes hydrolysis when heated (27) Our present concept is that each sample of inulin is composed of a number of variably sized molecules which give an average molecular weight This weight will vary with the source of the carbohydrate, and the procedure utilized for its extraction

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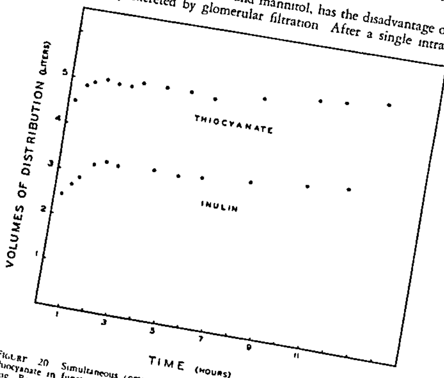


FIGURE 20 Simultaneous comparison of the volumes of distribution of inulin and thiocyanate in function of time after single intravenous injection in a nephrectomized dog Reprinted, by permission from Gaudino M, and Levitt, M F Inulin space as a measure of extracellular fluid *Am J Physiol* 157 397 (1949)

venous injection in animals with intact kidneys, its excretion is so rapid that uniform distribution throughout extracellular space does not occur Uniform equilibration after a single injection only occurs in nephrectomized animals.

Angevine· You say "rapidly excreted"; exactly how long does it take?

Gaudino. After a single injection, about 90 per cent of the amount administered is recovered in the urine; this occurs within three to four hours in man, and in about two hours in the dog

Figure 20 shows a simultaneous comparison of the volumes of distribution of inulin and thiocyanate in a nephrectomized dog As you can see, after two hours there is complete equilibration of inulin, and its volume of distribution is about a third smaller than that of thiocyanate.

In order to compensate for renal excretion of inulin in animals with intact kidneys, or in man, and to permit complete equilibration of this substance throughout its space of distribution, we devised a method by which a constant intravenous infusion of inulin is given for a long

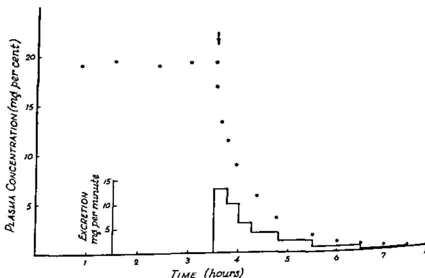


FIGURE
method,
fusion
discontinued, and the bladder emptied and ...
until the inulin has been completely excreted *Reprinted*, by permission, from Levitt, M F, and Gaudino, M Measurement of body water compartments *Am J Med* 9, 208 (1950)

time. This infusion compensates for renal excretion of inulin, and maintains a constant plasma level until uniform and complete distribution is attained (23, 24) Figure 21 illustrates a determination in the dog, and plasma concentration is seen to become constant after one hour However, constant plasma concentration is not a sure indication of equilibration. The infusion is continued until uniform distribution is established, then simultaneously (as indicated by the arrow) a blood sample is taken, the infusion is interrupted, and the bladder emptied by catheter and rinsed. Urine is then collected quantitatively until all inulin contained in the body at the time of the cessation of the infusion is excreted The lower part of the figure shows this cumulative excretion. The total amount collected, divided by the plasma concentration immediately prior to the interruption of the infusion, gives the volume of distribution of inulin.

When we first approached the problem of measuring inulin space in subjects or animals with intact kidneys, we asked ourselves the following question. why couldn't the amount of inulin contained in the body be estimated by the difference between the amount infused and the amount excreted during the course of the infusion? At that time a good apparatus for the administration of infusions at a constant and exact rate did not exist Consequently, the total collection of inulin excreted after the end of the infusion resulted in a more accurate estimation of the total amount of inulin contained in the extracellular space than calculating it by difference There are now on the market several infusion pumps of very precise rate, which permit this calculation to be made accurately The method of calculation by difference has the disadvantage that when the duration of infusion is very long, the difference between the amount infused and that which is excreted becomes so small with respect to the quantities themselves that this difference, equal to the amount contained in the body, falls within the error of the method As an example, if the infusion has been very long, 30 0 gm of inulin may have been infused, and 28 5 gm may have been excreted The body therefore contains 1 5 gm equal to 5 per cent of the total amount injected, and within the error of the whole procedure On the other hand, if the infusion has been moderately long, 6 0 gm of inulin may have been infused, and 4 5 gm excreted The body in this case still contains 1 5 gm but now this value corresponds to 25 per cent of the total amount injected, definitely greater than the error of the method The basic condition for accuracy with both procedures—the total recovery method and the method by differences—is that inulin should be completely recovered in the urine in each experiment, otherwise the measurement becomes invalid

Mirsky: Do you have to plot the infusion rate in order to obtain that plateau?

Gaudino: No, the infusion is either given constantly from the beginning, or a priming injection is administered prior to it The blood level will subsequently attain constancy, which will occur more rapidly if a priming injection is given.

Mirsky: I see, you compensate for it. Is there no limit to the amount of inulin that can be excreted?

Gaudino. As I mentioned before, there is a certain time required to achieve equilibration, that is, for inulin to diffuse into the whole of the extracellular space. We found that in the dog this time was about two hours, and in man about five hours. In more recent experiments of ours (28), the equilibration time in man was between three and four hours. This shorter time may be attributed to a probably lower molecular weight of the inulin utilized.

In man, sucrose can be used in the same fashion as inulin, and its physiological behavior is exactly the same, being completely recovered in the urine. It has the advantage of a more rapid equilibration, which is obtained in about one hour as compared to the three to four hours required for inulin (22).

Inulin space measured with this equilibrating infusion method averages 19 per cent of body weight in the dog, and between 15 and 19 per cent in man These values may be compared to 21 to 26 per cent for thiocyanate space, and 23 or 35 per cent for chloride space in man (10, 16) Sucrose, when used with this method, gives exactly the same space of distribution as inulin, i.e 17 to 18 5 per cent of body weight in man (22).* The [illegible] weight, and sucrose, [illegible] measure an identical [illegible] ing either total extracellular space, or an extracellular pool of water of great physiological importance. This claim is further strengthened by the fact that simultaneous determinations of ferrocyanide and inulin spaces in infants are the same in volume (29).

It is known that inulin, thiocyanate, and sucrose, do not diffuse into the cerebrospinal fluid in appreciable degree. This specialized space should not be considered as part of the rapidly exchangeable extracellular space.

Angevine. What is the explanation for the fact that sucrose damages cells so readily? It must get into the cell

Gaudino It has been reported in the literature that the intravenous administration of sucrose may produce hydropic degeneration of the

* Gaudino, M Unpublished observations

proximal tubules (30, 31, 32, 33) However, sucrose administered in physiological amounts does not produce permanent injury to the kidney, when it is given for the measure of renal function, or space of distribution No untoward effects or changes in renal function have been reported under those circumstances The reported damage to cells produced by the administration of sucrose was subsequent to very large doses of the very concentrated solution (30, 31) In this case, I assume that by acting directly outside the cells it might have produced secondary alterations in them.*

Angerine But in patients it is given in this concentrated form, I am quite sure of that

Gaudino When sucrose is used as a measure of extracellular fluid, or of kidney function, it is given at a very low concentration The amount and concentrations used do not seem to produce any pathological changes

Angerine Yes, but the fact that it hits the cell makes one wonder whether there would not be some diffusion into the cell

Gaudino· I think the facts are against the possibility of diffusing into the cell Pathological changes observed may be secondary to changes outside the cell Sucrose, when used as a measure of extracellular space, has the same volume of distribution as inulin This last substance, from what I have said previously, in all probability does not enter the cell There are no reported damages to any tissue subsequent to the intravenous administration of inulin

Now, returning to the discussion of inulin space as a measure of extracellular fluid, there is one possible objection which I have not mentioned previously It refers to the fact that as inulin gives the smallest space of distribution of all substances mentioned, it may possibly not have diffused into the totality of the extracellular space However, by prolonging the time or infusion in the same subject in different experiments, inulin space did not increase These studies have been done both in man and in animals In man, we have given up to 20 hours of infusion, and in animals, up to about 12 hours Inulin spaces of dis-

* Rigdon and Cardwell (32) have reported that the appearance of hydropic tubular lesions after injections of sucrose depends largely upon the state of hydration of the subject In well hydrated animals no lesions are observed even after very high doses, while in dehydrated animals lesions appear with lower amounts of sucrose The same applies to pathological findings in man Wilmer (33) concludes that lesions observed after the administration of sucrose are due primarily to its osmotic effects

Although more conclusive evidence should be brought forth, these results point to the fact that so-called pathological lesions may not be more than histological manifestations of the overactivity of the tubules, in response to an osmotic load imposed by the excretion of such concentrated solutions of sucrose This concept would be compatible with the postulated impermeability of cells to sucrose

tribution were identical whether the infusion was long or short, once the minimum time required for uniform distribution had elapsed Our criterion for uniform distribution was that any further prolongation of the infusion did not increase inulin volume of distribution That complete diffusion had taken place for inulin, is further strengthened by the fact that its space of distribution is identical with those of sucrose and ferrocyanide when measured simultaneously, seeming to argue in favor of the concept that they are all measuring a space of physiological importance.

Ragan: Is there any difference in the space between the inulin which takes six hours to equilibrate, and the one which takes four hours?

Gaudino: We have not utilized these different inulins in the same patient However, the average spaces obtained in the two series of patients given different types of inulins are the same.

Mirsky. That is exclusive of the cerebrospinal fluid?

Gaudino: Yes Of course, chloride exists in cerebrospinal fluid, and therefore must enter into it However, I do not think that its rate of entrance is known, and I do not believe that there are any studies reported about the entrance of radioactive chloride into cerebrospinal fluid either.

Ragan: Does inulin enter the synovial fluid?

Gaudino: I do not know, and I doubt if there are any such investigations reported in the literature. Of course, these studies could be done either by introducing inulin directly into the synovial cavity and finding its appearance in the systemic circulation, or vice versa by introducing it in the systemic circulation and then determining its rate of appearance in the synovial fluid.

Mirsky: But when you talk about space and water distribution, you are dealing with a larger volume, relatively speaking, than that of the cerebrospinal fluid.

Gaudino: Yes, that is true. The fact that inulin does not enter into cerebrospinal fluid should not be very important. However, this objection was raised so many times that we had to answer the point.

Mirsky: There is a large volume of cerebrospinal fluid

Gaudino: How much?

Fremont-Smith: Not more than 150 ml.

Gaudino: Extracellular space in man ranges from 10 to 15 liters I think that this answers your question.

Mirsky: Yes, I see

Gaudino· As a matter of fact, when inulin space is measured in infants it gives a much larger space of distribution per cent of body

weight than in adults. The range is from 21 to 33, with an average of 27 per cent (34).

Meyer: The answer which you just gave about children would immediately raise the question that this, by definition, becomes a rather arbitrary concept, because we can speak only of inulin space

Fremont-Smith. And chloride space.

Meyer. Let us assume that there are various degrees of binding of water in the interstitial spaces.

Gaudino: Yes, if you have accepted the hypothesis that there is binding of water.

Meyer. I believe we have to assume that there is bound water

Gaudino. What about diffusion of heavy water?

Meyer. I believe the bound water will not distinguish between heavy water and ordinary water

Gaudino. Do you mean that the fact of being bound would not prevent it from being exchangeable?

Meyer Yes. I presume it would exchange, just as water of crystallization exchanges

Gaudino What evidence is there that it is bound?

Meyer. For example, I saw a very interesting paper by Ernst (35), in which I thought he showed clearly that there is bound water in muscle

Sinex. Let us define bound water What do you mean by that?

Gaudino. It is a very elastic definition

Fremont-Smith I believe the authorities do not agree on a concept of bound water

Gaudino. Exactly According to what Dr Meyer is saying, bound water is water that, although fixed in place, can be molecularly exchanged But if there is bound water inside the cell, could a molecule of that water be exchanged for a molecule of water from the surroundings?

Meyer Yes, this is an exchange process

Gaudino Then it would not make any difference for our purpose whether or not it was bound water

Fremont-Smith. It would depend upon what the purpose was

Gaudino That is right Our purpose would be to measure the exchange of water, or the spaces of distribution of water

Meyer What other explanation can one offer that the space measured by inulin, sucrose and mannitol is so different from that measured by sodium or chloride?

Gaudino One of the answers might be that both sodium and chloride are partly contained inside the cells

Meyer: Yes, but you assume that sodium is also in some kind of equilibrium.

Gaudino. I have no specific assumptions about it In some recent work on potassium, it was demonstrated that this ion is not contained in a uniform concentration inside the cell; it accumulates in the mitochondria (36) It may be, therefore, that the portion of sodium and chloride contained inside the cells may not be in complete equilibrium with the outside, and may be accumulated in different portions of the cell In that case, they may be either bound or unbound. This is a problem that should be studied further.

Fremont-Smith: I have been brought up to believe that bound water is water not available as a solvent, and therefore not to be considered in measuring activity concentrations or osmotic concentrations. Is that still a useful definition?

Meyer. It seems to me we have to postulate fibrous structures which are hydrated. The forces binding this water must be very similar to those binding water of crystallization

Fremont-Smith: And therefore not available as a solvent.

Meyer If we admit that the structures are colloids, the water phase will bind certain ions without serving as water of solvation for inulin or sucrose

Fremont-Smith. Or of free ions.

Meyer: Yes. It will bind certain ions, sodium and chloride, for example, will be bound and exchanged.

Gaudino Therefore, this structure will permit the sodium and chloride to go through On the other hand, you do not think that inulin or sucrose would not be able to distribute through it?

Meyer No This bound water does not form a rigid layer, but may be thought of as a field of diminishing strength with the strongest binding forces close to the center, that is, the fibrous structures

Gaudino This is a good point and I think we have to consider it On the other hand, there are many tissues which contain so much chloride and sodium that one is forced to assume that a large amount of these ions exists inside the cells

Meyer. Is that sodium in bone and cartilage?

Gaudino. Yes. But what about liver? Chloride space in this tissue ranges from 25 to 35 per cent of weight, and sodium space from 30 to 38 per cent.

Meyer: I do not know about that.

Gaudino: In many tissues there does not seem to be great histological evidence for the existence of a large extracellular space, most of them seem to be composed of capillaries and cells. Perhaps the pathologists

here may be able to tell more about this problem. One of the problems we have been faced with is that of estimating the percentage of the total mass which is vascular volume for each type of tissue The data in the literature are rather scanty on this point

Fremont-Smith There is one interesting thing about the concept of bound water it is known to have a great deal of latent heat in it.

Gaudino. Not only that, but the existence of bound water makes the explanation of exchanges much easier

Sinex. A recent paper on the subject of ion exchange resins, by Wheaton and Bauman (37), is well worth reading Generally speaking, columns behave as if there are two water phases, one outside the resin particles and another within. When different solutes are introduced into the flowing phase of the column, they distribute themselves between the two water phases in a manner which is characteristic of the solutes The ability of such columns to fractionate complex mixtures does not necessarily depend on ion exchange, it may depend on the ability of the solutes to enter the bound water

Meyer In other words, we have the concept of a variable degree of binding of the water?

Sinex. We are going back to the ability of solutes to enter a particular water phase, if this water phase is associated with an insoluble charged electrolyte.

Meyer: But it is not

Sinex. Yes, it is.

Fremont-Smith In other words, we have to say that relativity enters in there also

Sinex. I would think so

Fremont-Smith. You do not have absolute binding or unbinding

Meyer I think it makes an enormous difference, because this space should be an extremely important space, it seems to me

Gaudino· It probably is And moreover, as a consequence, it would appear that instead of having two or three spaces one could have six, seven, or eight, and depending on the organs or tissues, one would have multiple spaces inside the cell Examples of subdivisions of the intracellular water space would be· mitochondrial space, nuclear space, and so forth This concept makes the problem much more difficult. So far, we have not even agreed on what we are measuring with substances like inulin, sucrose, and thiocyanate, and I do not know how we can even get into the other problem

Mirsky. Lack of agreement may merely be due to the fact that there is an excessive oversimplification

Gaudino: That is right. If we knew more about the distribution

of thiocyanate, which has in certain conditions rather stable spaces of distribution, and chloride or inulin simultaneously, one could separate different spaces and try to find what that difference between the two spaces is measuring. It would perhaps measure binding and lack of binding, or the existence of a certain amount of water, which although bound and not available as a solvent for inulin or sucrose, would still be available for chloride or other similar ions

Fremont-Smith. Could one say that this is the concept of intercellular spaces, or is that an oversimplification?

Gaudino: No, it could be.

Fremont-Smith. The particular purpose is to show wherein the oversimplification lies; these are temporary expedients in order to come to grips with the problem and find out where we are wrong If we approach it from that point of view, then the latent heat seems to disappear from it.

Gaudino Of course, in this problem we have been dealing with spaces in quite a broad manner We seem to have thought of the body as a big water bag, with one or two smaller water bags inside; we measured the changes that occur in all the bags, mostly determining just increases or decreases We do not know if there are any organs or tissues which may act as reservoirs of water for the organism, or if that water accumulates in some tissues with preference to others when increases occur Most studies which have dealt with tissues have been done in muscle, but muscle is not the whole body and much still remains to be done

Holbrook But it appears that due to the similarity of your results, you are at least measuring some water space, whatever that is

Gaudino We call it inulin space. Someone said that inulin space measures inulin space!

Holbrook That is fair enough.

Gaudino. Assuming that what we have measured is what we think we have, then with an accurate determination of extracellular fluid volume we can do several things. First, by simultaneously measuring total body water with deuterium or tritium, we can estimate intracellular water By also measuring plasma volume we can estimate the volume of interstitial water Utilizing any of the radioactive isotopes of chloride, sodium, or potassium, and assuming complete exchangeability with the nonradioactive form, we can calculate total chloride, sodium, or potassium mass in the body. If the total mass of the element is assumed in solution, and at equilibrium with plasma, then the volumes of distribution may be estimated. Coupled with the determination of

total mass of the element, an accurate extracellular space estimation will permit the calculation of the proportions of each ion which are extracellular and intracellular

I should like to present some experiments we have done in the last few years that show rather rapid changes in the inulin space. In all of them, plasma volume was measured simultaneously with inulin space, and while the former did not change appreciably, the latter was observed to vary quite markedly Therefore, in the following presentation, changes in the inulin space will reflect almost exactly changes in interstitial fluid volume

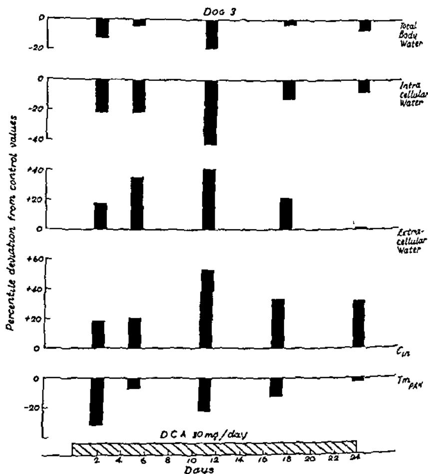
At the beginning we were interested in studying alterations in body water compartments determined by the administration of adrenal hormones and by adrenalectomy (38) In a group of normal dogs we investigated the effect of the subcutaneous administration of 30 mg. of desoxycorticosterone acetate (DCA) per day Several space determinations were done before and during therapy I might mention here that the measurement of inulin space, by means of a constant equilibrating infusion, permits the simultaneous estimation of glomerular filtration rate and renal plasma flow if *p*-aminohippurate (PAH) is added to the solution

In Figure 22, the abscissae indicate average control values which did not vary more than from four to six per cent Percentile changes observed in some of the functions studied are shown as black bars. The top graph describes modifications in total body water, as measured by the space of distribution of D_2O The third graph indicates changes in inulin space, while the second shows calculated variations in intracellular water as obtained by difference between D_2O and inulin spaces. The last two graphs depict variations in glomerular filtration (inulin clearance) and Tm_{PAH} The hatched area indicates duration of the treatment with DCA

It can be seen that as a result of the treatment, inulin space increased progressively up to the 11th day and then, in spite of continuation of therapy, it began to revert to normal On the other hand D_2O spaces either remained approximately at control values or increased slightly As a consequence intracellular [illegible] markedly With [illegible] proportionately [illegible] There was no [illegible] animals, mod[illegible] [illegible] as a consequence of [illegible] creased progressively, reaching a maximum approximately on the 11th day, and then tended to return to normal despite [illegible] DCA administration, in some cases even at a higher [illegible]

Ragan: What levels of serum potassium were reached?

Gaudino: In these animals, plasma potassium fell to approximately half of its control value, that is, from 4 to about 2 mEq per liter, for about three weeks.



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Ragan: Was the sodium going into the cell?

Gaudino: I am not certain. However, plasma sodium levels did not change appreciably.

Ragan. Ferrebee (39), in 1941, showed that there was an increased intracellular sodium in skeletal muscle after DCA treatment

Gaudino: That might very well have happened.

Ragan: Did your dogs develop any diabetes insipidus or paralysis with 30 mg. a day?

Gaudino: We did not observe any development of diabetes insipidus, although we did not look carefully for its manifestations But I am

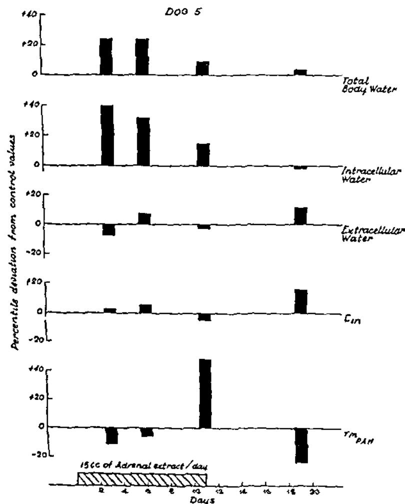


FIGURE 23 Action of adrenocortical extract on body water distribution and renal function Reprinted, by permission, from Gaudino, M, and Levitt, M F Influence of the adrenal cortex on body water distribution and renal function *J Clin Investigation* 28, 1487 (1949)

sure that they did not show any paralysis. They were perfectly normal-looking animals.

Ragan: Did you feed them meat?

Gaudino. They were receiving the usual dog food, but no meat In one of these animals we estimated total body sodium with Na^{24}. We found an increase in total sodium mass in measurements made on the 16th and 23rd days of therapy. This increase disappeared and values reverted to normal 30 days after discontinuing the injections Intracellular sodium increased from control values of 170 mEq, to 284 and 304 mEq. on the 16th and 23rd days of treatment respectively. It reverted to 210 mEq 30 days after stopping therapy. In one dog, measurement of total body potassium with K^{42} showed both a decrease in total and in intracellular potassium at the 11th day of therapy.

The effect of the administration of 15 ml of adrenocortical extract (from the Upjohn Laboratories) per day is shown in Figure 23 It may be observed that while there was little or no change in inulin space, total body water increased, resulting in an increment in water up to about 40 per cent of its original level Both glomerular filtration rate and renal plasma flow were not significantly modified. Tm_{PAH} varied in an irregular fashion No significant changes were observed in plasma sodium, potassium or nonprotein nitrogen In this case, it was also observed that with prolongation of treatment, modifications observed tended to revert to normal

Figure 24 gives the results of experiments on a completely adrenalectomized animal, which was maintained with 15 mg per day of DCA until 16 days after the operation. It is interesting to note that while under treatment no signs of adrenal insufficiency developed, and when measurements were performed during this period, the animal showed similar changes in spaces and renal function as those observed in normal dogs receiving DCA, i e., increase in inulin space, a slight decrease in total body water, and marked decrease in intracellular space After DCA injections were interrupted, measurements were done on the 4th and 7th days of insufficiency. Inulin space progressively decreased, while total body water did not change, resulting in a marked increase in intracellular water Changes observed in this animal became more conspicuous as the degree of adrenal insufficiency increased, alterations in body water compartments being the most prominent features Inulin space declined 39 to 61 per cent, while total body water did not change Intracellular water increased up to 30 per cent of preoperative control levels It therefore appears that as a consequence of adrenal insufficiency there is a prominent increase either in the intracellular space or in the

space not available to inulin In this particular animal, after the 7th day of insufficiency and after the second group of measurements, DCA treatment was resumed at a dosage of 10 mg per day In spite of this, modifications in water compartments, although less intense, did not revert to normal The animal died after the last measurement.

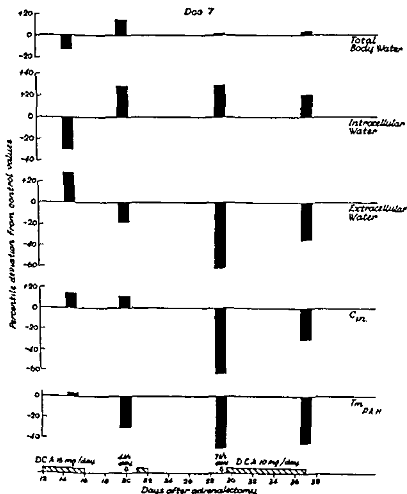
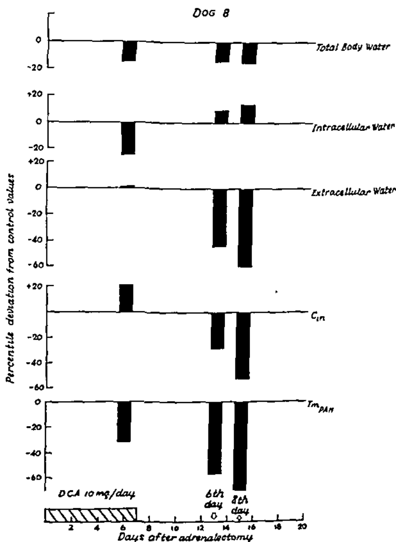


FIGURE 21 Changes observed in water distribution and renal function after complete adrenalectomy in the dog Reprinted by permission, from Gaudino, M, and Levitt, M F Influence of the adrenal cortex on body water distribution and renal function *J Clin Investigation* 28, 1487 (1949)

Figure 25 is another example of the effect of total adrenalectomy in the dog During treatment, changes observed were not as marked as in the one previously shown, although still similar to normal dogs receiving DCA After interruption of treatment, disturbances in body water distribution became quite marked, and were analogous to those



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shown in Figure 24 After the 8th day of insufficiency, the animal died at the conclusion of the second experiment As may be observed in both Figures 24 and 25, renal function showed marked changes glomerular filtration, PAH clearance and Tm_{PAH} were all ... progressively reduced The ... insufficiency were preser... sodium decreased moder... nitrogen rose, while the specific gravity and the hematocrit increased. Blood pressure was reduced, particularly in the terminal stages These alterations were more conspicuous with increased severity of insufficiency It is interesting to note that in these adrenalectomized animals plasma volume remained at control values during mild degrees of insufficien... the other water compo... 35 per cent in the ... more markedly in ... its absolute decrease, correspon... about one-half to three-quarters of the inulin space It appeared as if in adrenal insufficiency, plasma volume and intracellular volume were maintained at the expense of interstitial space

Similar studies were done in man by Levitt and Bader (40) after the administration of cortisone or ACTH These hormones were given daily for periods from 10 to 20 days, cortisone at a dosage of 100 to 150 mg, and ACTH at a dosage of 100 mg The subjects were kept on a low salt (rice) diet Administration of both cortisone and ACTH markedly increased inulin space, while plasma volume did not seem to change Consequently, interstitial space, or space available for inulin distribution, was conspicuously enlarged Plasma sodium and chloride concentrations did not vary from control values, indicating an isosmotic expansion of interstitial space

Dr Crawford and I (28) have done some studies in humans on the effect of intravenous saline solution administration, and of short periods of anesthesia, on inulin space and renal function Inulin space was measured with both the postinfusion recovery method described previously and the calculation by difference After several control inulin spaces were obtained, isotonic saline solution was infused intravenously at the rapid rate of 40 to 60 ml a minute, in amounts of 2 to 3 liters Space measurements were repeated one and two hours after saline administration was completed In the majority of the patients studied, the results of the 20 tests showed an increase in inulin space This increase was 1 to 4 liters larger than the volume of saline solution administered in 35 per cent of the subjects In 20 per cent it was equal,

and in 30 per cent, less than the volume infused. In only 15 per cent of the cases, inulin did not vary from control levels.

Mirsky: Suppose, by that mechanism, that you increased the reabsorption of water, would that affect it?

Gaudino: Do you mean that there would be an increased reabsorption of water by the kidney?

Mirsky: Yes.

Gaudino· I do not think this mechanism would influence the measurements very much We have measured water excretion and water reabsorption in these subjects, and did not find any significant modifications After the administration of saline solution, urine flow always increased, but markedly in only very few cases In those patients who received 2 liters of saline, the average recovery of water in the first two hours following the infusion was about 13 per cent, while sodium recovery averaged 22 per cent In patients receiving a 3-liter infusion, water recovery after two hours average 18 per cent, and sodium, 23 per cent Therefore, there is still no explanation of why 2 liters of saline solution administered led to an expansion of up to 6 liters in inulin space In 50 per cent of the subjects who received short periods of anesthesia alone, and no saline solution intravenously, inulin space increased from 1 to 7 liters, which corresponded to a 6 to 83 per cent increase from control inulin spaces

Ragan. If there was a 1 to 7 liters increase, would one obtain a 7-kilo increase in weight?

Gaudino: These patients did not receive any fluids; therefore, these values correspond to internal shifts of water

Ragan: This is a shift, but from the intercellular space, do you obtain 7 liters?

Gaudino: Yes, that is right.

Meyer: Have you attempted to explain that?

Gaudino· The value of 7 liters is actually an extreme figure. The average change was between 2 0 and 3 5 liters These patients did not [illegible] anesthesia,

200 to 300 ml This amount of water introduced in such a long period of time does not seem to alter inulin space in a detectable manner Anesthesia was maintained from 30 minutes to 3 5 hours, and no fluids were given intravenously during this period Measurements of the extracellular space were started immediately after anesthesia was finished, and in no case later than 30 minutes after. Therefore, in spite of the lack of fluids, inulin space expanded in about half of the cases Evi-

dently, there must have been a shift of water from the cells into the inulin space, or according to the thinking of Dr. Meyer, an unbinding of water which then became available as a solvent for inulin

Fremont-Smith. Was there sweating, or any other loss?

Gaudino. Yes, in some subjects there was a certain amount of sweating, which would have dehydrated them However, I do not think that the amount of sweating was sufficient to modify water distribution. Sweating would have tended to decrease the water content of the body, rather than increase it.

Meyer: How much change did you have in the tonicity of the extracellular space?

Gaudino. Plasma concentrations of sodium, chloride and potassium did not change significantly in these patients, therefore, no great change in tonicity seems to have occurred.

Fremont-Smith You do not quite know what comes out of the cells, do you?

Gaudino No, I do not, and I do not know either whether electrolytes come out from a special organ or a special area in the organism We are dealing here with the big water bottle, and in this case we see large changes only There may be compensatory alterations which pass undetected, and there might be localized modifications appearing as changes in the whole body.

Fremont-Smith Have you ignored the fluid in the gastrointestinal tract?

Gaudino Yes, we have.

Fremont-Smith How large an amount is that?

Gaudino. I do not know But even taking into consideration gastrointestinal tract content in patients who have not been given anything by mouth, or intravenously, for eight hours, one could not explain the changes Furthermore, the amount of water contained in the intestinal tract probably does not vary significantly with anesthesia

Holbrook There certainly is no increased absorption in the gastrointestinal tract with anesthesia, it would tend to be the other way.

Fremont-Smith But there may be continuing absorption, there is water available there For instance, has bile or pancreatic juice been poured into the gastrointestinal tract? Water is being absorbed from the lower bowel continuously, is it not?

Gaudino I think that some fluid might be absorbed that way, but I do not know the quantity

Fremont-Smith. I was wondering whether you ought to mention this as an unknown compartment which has not yet been measured.

Gaudino It is actually separated from extracellular space by a con-

tinuous layer of cells, and therefore should be considered more as a type of spinal fluid.

Holbrook: But the same water is in the bowel when these experiments are performed, and you do not obtain a 3-liter increase

Gaudino: That is right. Our main point was to study what happened to the inulin space, and we observed that it increased.

Holbrook. I should think it might be increased even more than your figures show. If anything, the error is on the other side.

Gaudino: Yes. And I may mention here that in a somewhat similar type of experiment, Levitt and collaborators (41) observed an increase of 1.5 to 2.0 liters in the inulin space of subjects in whom tourniquets were applied to both legs for from one to three hours. This expansion of extracellular space corresponds to about 16 per cent of control values

Fremont-Smith: In the legs?

Gaudino: No, in the whole body.

Ragan: After the tourniquets were removed?

Gaudino. No, during the application of the tourniquets

Mirsky. It is interesting that all the procedures you have described so far result in a tremendous release of vasopressin

Gaudino: Do you imply by your statement that there is a vascular component to these changes?

Mirsky. I am just mentioning a fact; and am trying to see how it fits in with this change

Gaudino. Does DCA increase the release of vasopressin?

Mirsky: I know of no better way to increase vasopressin in the circulation than by the injection of saline in that volume, anesthesia, and the application of tourniquets

Holbrook: Not DCA, though

Mirsky. I would not be surprised if DCA were equally effective. Of course, adrenal insufficiency, and also marked loss of fluid, will have the same effect.

Gaudino: But in adrenal insufficiency there is a decrease in inulin space.

Mirsky: I'm not sure that DCA does not do it

Gaudino: In Levitt's experiments, after the increase of inulin volume of distribution determined by the application of tourniquets, plasma levels of sodium and chloride did not change. Therefore, there was an isosmotic expansion of inulin space, indicating a movement of both water and electrolytes

The rapid changes observed in the water, sodium, and chloride of [illegible]tial spaces bring up the following question: where does all

this water, sodium, and chloride, come from? It may be assumed that water comes from the cells, and perhaps from the gastrointestinal tract; sodium may be supplied by connective tissue, bone and the cells, and chloride, probably from both the cells and connective tissue However, all this is speculation, and the problem of finding these sources is still an open one.

Sinex. What about potassium in connective tissue? Can we assume that all the extracellular cation, associated with the extracellular phase of connective tissue, is pretty much all sodium?

Gandino: The general assumption is this. interstitial fluid is in osmotic equilibrium with plasma Plasma, on the other hand, contains only very small amounts of potassium, therefore, interstitial fluid also contains a small quantity of potassium, and at a concentration practically equal to that of plasma water We may now take muscle as an example of whole tissue It is known that about 16 per cent of its mass would contain all chloride found by chemical analysis, in the same concentration found in plasma water Chloride presumably being extracellular in muscle, it follows that about 84 per cent of muscle volume corresponds to cells If potassium in that 16 per cent extracellular space is considered to be at a concentration similar to a plasma ultrafiltrate, then the amount of potassium contained in that volume is only a small percentage of the total potassium contained in muscle Applying the same considerations to sodium, the opposite result is found, from which it is concluded that sodium is the principal extracellular cation

Meyer Do you know whether ionized calcium is higher in the connective tissues?

Gandino. I do not know much about calcium in tissues, and I think not very much is known about magnesium, either From all these considerations it appears that one of the most important, and less known functions of the interstitial spaces, is that of being a buffer compartment, a depot, and an avenue of exchange for water, electrolytes, and other substances necessary for the metabolism of the cell, or resulting from this metabolism If interstitial space is considered to be composed mostly of connective tissue, this would then appear to be one of the main functions of connective tissue

There are now a few questions I should like to ask Which part of connective tissue is concerned with the function of storage, and the exchange of electrolytes and water? Is it the fibers, or the ground substance? How do the known morphological components of connective tissue (fibers and ground substance) and the chemical components (mucopolysaccharides, collagen, and so forth) enter into the picture? What function may some of the enzymes concerned with composition

and normal condition of connective tissue have in fluid and electrolyte balance?

Finally, there is the question of how the histological picture of tissues and organs agrees with this function of connective tissue Is there a histological counterpart for the spaces between vessels and cells in the amount that we are measuring? Is it true that between 13 to 16 per cent of body weight corresponds to that histological interstitial space? This is a problem which is interesting, not only to physiologists and clinicians, but also to pathologists. We are here measuring a space which is considered to be of physiological importance for the exchange of water and electrolytes, and are assuming that it has an anatomical counterpart But this counterpart has not been very thoroughly studied. I do not know how accurate the estimation of interstitial space between cells is in frozen sections of muscle, or whether similar studies have been done in other tissues and organs of the body. Perhaps Dr Angevine will give us an answer.

Angevine. I think one would have to do some planimetry, or one can weigh muscle fibers, and the interstitial tissue, by historadiography

Gaudino: How is that done?

Angevine: They can be weighed by using a historadiograph, and making densitometer readings to determine the densities of the interstitial tissue and the muscle in relation to a standard reference system.

Gaudino. Isn't that a somewhat uncertain method? I should think it might be difficult to differentiate between what is extracellular, and what is intracellular.

Angevine: Yes, but whatever answer one would obtain histologically, it would be just a general trend rather than a very accurate determination.

Meyer. Some very interesting work on the waterbinding of a connective tissue has been done with the cornea, which probably is one of the best tissues for the study of such problems. It swells *in vivo* and *in vitro* under a variety of conditions. Apparently it does not shrink unless exposed to heat It will swell in hypertonic and hypotonic solutions, on changes of pH, anoxybiosis, and under the influence of certain poisons which interfere with the energy metabolism, as shown recently by a British author, Dr M. Langham (42). The water uptake is particularly limited to the *substantia propria*, which is made up of collagen fibrils and certain mucopolysaccharides, among which sulfated polysaccharides predominate.

I have pointed out previously that there seems to be a biological antagonism between hyaluronate and the sulfated mucopolysaccharides Where hyaluronate predominates, or is the only polysaccharide present,

and normal condition of connective tissue have in fluid and electrolyte balance?

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one finds edema, as in myxedema, in the sex skin of the monkey, and presumably in the retrobulbar tissue in exophthalmos The tissues containing predominantly or exclusively sulfated polysaccharides have a low water content Thus, the question arises whether and how we can relate changes in water content, or hydration of connective tissue, to changes in their structure. I believe that we have to assume that when connective tissue swells, the water content of the ground substances rises primarily, and the collagen fibers become hydrated secondarily These shifts in water binding may be initiated by interference with the energy metabolism.

Gaudino. In these cornea studies you just mentioned, how can you explain the metabolism, the source of energy, if you say that there are so very few cells?

Meyer. Nuclei may not be necessary for metabolism Furthermore, a few cells may regulate the functions of quite a large area, as in muscle

Gaudino Do you mean to say that the ground substance *per se* is alive in the same way that a cell is alive?

Meyer I think it is erroneous to assume that all the energy metabolism has to be bound to cell structure

Gaudino Therefore, another way of considering the problem would be to assume that the ground substance, intercellular substance *per se*, would have a metabolism of its own.

Meyer Yes Just as in muscle, these few cells regulate the function of quite a large area Where the ground substances are formed, we do not know We have to conclude from the work with S^{35} sulfate of Bostrom (13), Dziewiatkowski (44), and others, that at least some of the mucopolysaccharide fractions are continuously reformed We assume now that the sulfated mucopolysaccharides are linked by weak forces to the fibrous elements This linkage is rather labile and may require energy to maintain, perhaps in a fashion similar to that required to maintain potassium inside the cell

Sinex One of the things we are considering now is whether collagen should be thought of as a more or less inert material, or whether it may have some of the properties of an organic catalyst bordering on an enzyme

Meyer This may be so I think, however, that the collagen fibril does not furnish the energy, it provides some locus, or loci, to which the ground substances are bound I would assume that this bond is a coulomb bond, or another weak bond In the ionized medium in which these structures are bathed, it presumably requires extraneous energy to maintain the structures intact

Gaudino. This is a rather interesting concept I think I mentioned

something before about the work of Mudge (36, 45, 46), in the United States, and Robinson (47, 48), in England. They found that by changing the metabolism of the cells, that is by passing from oxybiosis to anoxybiosis, there is a change in the rate of entrance of both water and electrolytes, especially potassium. This concept, therefore, could also be applied as you have just mentioned, to the ground substance and interstitial tissues.

Meyer: It can restore the water.

Holbrook: Dr. Meyer asked whether hydration of fibrous tissue might be the answer to this increase in space.

Gaudino: That was part of the question I put to Dr Angevine, on the proportion of these tissues in the whole organism. There seems to be very little data as to what percentage of the total body mass each tissue, or system of tissues, corresponds. It would be interesting to have such information for fibrous tissues, for the muscular system devoid of fibrous structures, and for cutaneous and subcutaneous tissue

The problem of interstitial hydration could be approached as long as we could measure inulin space *in situ* in the tissue, or any substance that would be exclusively extracellular in distribution. This would be particularly important in the case of a fibrous type of tissue, like tendon. Inulin determinations in tissues have been done, but they have the drawback of very high and somewhat variable tissue blanks, requiring very high blood levels (49) There are many tissue carbohydrates that give the same chemical reaction as inulin.

Sinex: C^{14}-labeled tubers have been grown at Brookhaven by Dr. Martin Gibbs for the synthesis of labeled inulin. This was in collaboration with Dr. Fabian Lionetti of Boston University

Fremont-Smith: In a patient with chronic edema, such as a cardiac or a nephritic, if we were to use a given edema level as the base line, and then make shifts in the compartmentalization of fluid, comparing it with shifts in an organism that is nonedematous, would it give us a clue as to what is going on? It would seem fairly clear that the gross edema of the fluid, or rather the edema fluid of gross edema, is not hydration; that fluid is not hydrated Therefore, if we were to make the assumption on the one hand that the fluid in the interstitial spaces is largely hydrated, as opposed to the other assumption that it is largely nonhydrated, and then make a comparison of the shift at that level with the shift when we knew it was largely nonhydrated, would we obtain deviations which would be significant?

Gaudino This might very well be an approach However, I do not think I mentioned before that one of the shortcomings of the inulin method is the fact that it cannot be used in patients with edema

Complete equilibration in this case requires such a long time that even if it could be achieved, the error involved in the calculation from the large figures of amounts infused and excreted, and from the small figures of amounts contained in the body, would be so great that it would be almost impossible to obtain an even approximate measurement Several investigators have tried to utilize this method in patients with edema and have failed

Fremont-Smith You could use chloride, though, could you not?

Gaudino Yes, one could use chloride in cases with edema Reports in the literature indicate that there is a definite increase in radioactive chloride space in patients with edema (10)

Holbrook Would you find the inulin space reduced in a patient with generalized edema?

Gaudino No, it is usually increased But sometimes in cases of large edemas, complete equilibration is not achieved, and therefore one is not able to measure accurately that enlarged space. In patients with moderate edema in whom complete equilibration has been achieved, there is an enlargement of inulin space (39)

Holbrook: But never to the degree that the edema exists, unless one waits long enough?

Gaudino Yes, but it would take perhaps several days to achieve equilibration

Fremont-Smith Do you mean that the inulin space can be increased almost as much as the gain in weight?

Gaudino Yes, I do not know whether actual measurements have been made, but I do not believe that there should be any difference between inulin space and increase in weight, as long as that increase in weight is due to an enlarged extracellular space

Meyer In cardiac or nephrotic edema, I believe there is a different problem It appears that normal skin contains no interstitial fluid, while in cardiac and nephrotic edema there is interstitial fluid in skin and loose connective tissue

Gaudino: With regard to normal skin not having interstitial fluid, do you mean in fluid form?

Meyer. Yes, in fluid form, I believe all experts in the field are in agreement on this Recently there was an article on this subject by Persson (50)

Gaudino With reference to skin, if one considers the total chloride or sodium found per unit weight of skin as extracellular, and calculates the spaces of distribution on the assumption that these elements are at a concentration equal to an ultrafiltrate of plasma, one finds that these spaces are either equal to or larger than the total water content of the

tissue Chloride space in skin can be between 60 and 70 per cent of its weight, while its total water content may be between 58 to 67 per cent. How can this fact be correlated with what you have just said?

Angevine: One can have water in a gel which is perfectly capable of holding sodium.

Gaudino: Yes, that may be so.

Sinex. I think you would find Loeb's book (51) on the swelling of gelation a very informative thing It was published in 1922, but it still has a lot of useful information in it.

Gaudino. Yes, I have read it.

Asboe-Hansen. I think myxedema would be another subject for research, but we seldom see untreated cases because the practitioners treat them before they come to the hospitals Could one find untreated cases in New York?

Fremont-Smith. One would find them here only occasionally, I think, when treatment was deliberately withdrawn for a while, or in the case of some patient who has had very ineffective medical care.

Travell: It might be found in a patient who has had a thyroidectomy, perhaps.

Fremont-Smith. Yes. I think it is very interesting that the same argument about bound and unbound water, gel and free-floating water in the skin, was taking place with considerable acrimony in 1924 and 1925 when I was first working with the cerebrospinal fluid, and it is still an unsettled problem.

Gaudino: There are many more measurements and exchange studies that should be done at the tissue level. If those changes were known, probably many things could be clarified As Dr. Meyer just suggested, if there is an increase in the amount of water contained in the skin, one does not know whether it is an increase of water of hydration of a gel, or just fluid water, and whether this increase occurs at the same time as a commensurate increase in the sodium and chloride content Were there any chemical analyses done on those skins that you mentioned, Dr Meyer?

Meyer. I do not know.

Fremont-Smith. It seems to me that that is a very difficult point to maintain unless we say that the skin of the feet, when we are in an upright position, is always abnormal We know very well that a considerable quantity of fluid from the blood stream goes into the subcutaneous tissues, which I assume would include the skin, although I may be wrong.

Ragan: But is it fluid as water, or fluid as gel?

Fremont-Smith: If it is fluid as gel, how rigidly do we mean gel?

Do we mean gel which behaves osmotically and otherwise like water, or do we mean gel that seems to be removed from the system altogether and set aside?

Ragan: Dr. Meyer says there are many gradations.

Gaudino: I may add that we are only speaking of interstitial tissue spaces If we go back to what was discussed earlier, and include the condition of vessels, capillaries, arterioles, lymphatics, and in addition to that, the state of the cells, all varying according to the part of the organism being considered, I think that the whole problem becomes almost insoluble.

Sinex: Some interesting information may come out of our work on the amino acid content of skin juice, because here we have a number of molecules of varying charge and size occupying a tissue which has a large extravascular component The free amino acids of the skin juice are definitely quite different from those of plasma

Gaudino Do they differ from those of tendon?

Sinex I do not know, I have not looked at tendon or muscle, but [illegible]

[illegible] That means you have [illegible]

Sinex: I have tried, but I do not know how thorough a job I have done

Gaudino: What do you do?

Sinex I heparinize the rats, take off the skin, pluck out the fur, and try, very grossly, to clean up the subcutaneous tissue Then I put it in a hydraulic press and rack it up to about 10,000 pounds. From the skin of a 150 gm rat I can sometimes obtain 2 ml or more of juice

Gaudino: I wonder whether you are certain that you have removed the cutaneous muscle which is so adherent to the skin of the rat

Fremont-Smith: How much of it comes from the cells? You certainly destroy cells, do you not?

Sinex. That is right, it brings up the question of what is the ionic composition of such juice The ratio of sodium to potassium is approximately 1 9

Gaudino You are obtaining extracellular fluid too, are you not?

Sinex Yes, I am sure about that

Fremont-Smith And all the gel fluid, bound, unbound, or partly bound

Sinex: That is right, it is a very complicated material that I have

Gaudino: Skin studies, especially in animals, should be done very carefully In the rat, and also in the rabbit, the dermis and the epi-

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Gaudino. Are you going to refer your results to wet weight of skin, to solid weight, or to nitrogen content?

Sinex: First, I should really like to solve the hydroxylysine problem

Fremont-Smith: You wish to know whether or not it is there?

Sinex Yes, I think that is my first problem; in fact, I am not too sure how far I would go with the second question I think the first problem is of considerable value to anyone who wishes to study the interaction of small charged molecules With the extracellular phase that exists in collagenous tissue, tendon or a thick layer of the skin may be utilized The juice can be pressed out and studied, and due to the fact that there are so many amino acids of varying molecular weight, something more may be learned about the actual environment in which these amino acids are found

Gaudino One of the important problems still unsettled is the existence, or nonexistence, of bound water in tissues This would be a fruitful subject of study for chemists, biochemists, or biophysicists However, they do not seem to be interested in the problem Do you agree, Dr Meyer?

Meyer This is a field in which the angels fear to tread, and I personally would not venture to work in it My purpose in discussing these very complex problems is to state my opinion that not all the questions have been solved Perhaps our concepts in this field are not on very firm foundations

Angevine In this connection, I think the studies of Dr Opie (52, 53, 54), in relation to osmosis, are of interest It was known, previously, that certain changes had to take place in the cell But in the technique he describes, water passes readily back and forth through cells and nuclear membranes

Gaudino: There are many indications that the cells are not in osmotic equilibrium, but what sort of equilibrium it is, we do not know

Fremont-Smith One has to make a distinction between water entering a cell, and entering a tissue There is a greater amount of water in the tissue afterwards; water enters a cell and leaves it in a back-and-forth movement which does not necessarily change the volume at all I think perhaps what Dr. Opie was referring to was the movement back and forth across the membrane

I had a difficult time trying to understand hydrostatic and osmotic pressure, and all of a sudden part of it seemed to come clear For instance, if there is a situation where there is water and a vacuum or gas phase, the water will gradually come into equilibrium with the water vapor In the beginning, more water molecules will escape into the vapor phase than will be coming back in, and when it reaches

dermis are closely adherent to the cutaneous muscle, which is quite developed in these animals. Therefore, if a careful dissection is not made, it is probable that the skin sample will have a large amount of cutaneous muscle mixed with it, introducing a large source of error A good dissection of the dermis and epidermis from the cutaneous muscle in these animals is not a simple procedure.

Angevine: One might squeeze out 2 ml. of juice from the blood vessels and lymphatics alone

Sinex. Yes, that's right, one could However, this juice is very rich in amino acids The concentration is five times that of plasma If we assume that the concentration of amino acids in the extracellular fluid is the same as in plasma, we might calculate from the sodium/potassium ratio that the true intracellular concentration might be as high as 70 µM per L, three times that of liver. Perhaps it is

Angevine A chemist uses all the precise methods of chemistry when working with tissues, but never seems to worry about what he starts with

Gaudino: Dr Angevine, it has been reported that in the muscle, the amount of blood contained in a sample of tissue can be measured by soaking it in saline solution, counting the red blood cells found in that solution, and comparing it to the red cell content of peripheral blood With this method, the amount of blood per unit muscle weight is about one per cent (49).

Sinex I have a lot to learn about dissecting layers of skin It might be better to use a larger animal than the rat.

Gaudino The skin of the rabbit is also rather difficult to dissect

Travell I should think there would be no shortage of human skin, if you are interested in that

Sinex: No, there is no shortage of human skin, but we cannot set up all our techniques at once I had very definitely intended to talk to somebody about preparations, I think the best suggestion is tendon

Gaudino: Do you make your analysis by paper chromatography?

Sinex: Yes.

Gaudino Are you particularly interested in content in muscle?

Sinex: I got into this by looking for free hydroxylysine phosphate and hydroxylysine For this purpose, I think one does not need to have pure dermal juice I should just like to know whether they are present in a tissue which contains a lot of collagen

When we approach the other problem of what is the amino acid composition of a particular layer of the skin, and how this is influenced by its immediate extracellular environment, we need a very carefully dissected layer, as you point out

Fremont-Smith That would increase the molecular concentration inside the tissues

Gaudino These tissues were soaked in saline for ten minutes I may mention here that people who do tissue culture work usually soak the tissues for half an hour to an hour in Tyrode's solution before putting them in the tissue culture medium, and that does not seem to alter their vitality to any extent

Fremont-Smith But it does not prove that many more small molecules have not been produced out of large molecules in the interval.

Ragan· Within ten minutes, the pH of the tissue is certainly much lower

Gaudino: Yes, the pH can change definitely Dr Opie has not analyzed either the tissues or the medium

Fremont-Smith The change would inevitably be one of increasing the osmotic pressure of the tissues that have been removed from the body I think what interested Dr Opie was this change

Gaudino There is a recent paper published by Deyrup (55), following the work of Drs Mudge and Opie, in which studies were done on the water uptake of kidney slices in the presence and absence of oxygen Incubation of these slices in the presence of oxygen determined the loss of the excess water taken in anoxybiosis Kidney slices incubated in isosmotic mediums, gained water if they were then incubated without oxygen, while they lost that excess water when incubated in oxygen In tissues stored from four to eight hours at room temperature, and then incubated, increase in weight was much larger than in the ones that had not been stored, however, in the presence of oxygen, they lost water as rapidly as the control tissues From these experiments, it appears that there is some relationship between metabolism, oxygen consumption, and water uptake in tissues I do not know whether these experiments may answer the point that the increase in metabolites of small molecular weight may produce an increase in osmotic pressure The fact of a loss of excess water after incubation in oxygen may be explained by an elimination of metabolites, or by their inclusion in larger molecules when normal metabolism is resumed

I recently started several experiments on this particular problem, and observed that after only two minutes of immersing tissues in their own plasma, significant gain in weight took place, a gain due entirely to water uptake by the tissues At the same time, potassium decreased to almost one-half its control values, while sodium and chloride increased quite markedly.

equilibrium, there will be an equal movement in both directions, provided the temperature is not changed. The degree of this movement is very great, even though there are no changes taking place

We shall assume that there is a membrane, and we shall create an osmotic situation by putting water in at one side, taking off this gas phase for the moment, and putting water there also, so that now we have water on the two sides, and a membrane which is permeable to water Obviously, in that case, water will be moving in both directions So far as I know, there has never been a theoretical calculation of how much water passes through, but the magnitude must be very great indeed

Now, if glucose is introduced into any molecule, there will be a movement of water It will rise up, which is the ordinary osmotic situation So far as I can understand it, the glucose has merely decreased the movement of water out; it has interfered with the escaping tendency of water across the membrane in one direction, and therefore has shown what movement of water occurred there in the first place The glucose will reduce the vapor tension, and so prevent the escaping tendency of the water into either a vapor phase, or across the membrane The movement which balances its going across a membrane, or across the vapor phase, must be of enormously large magnitude, and what we measure in osmotic pressure is merely the reduction of it, or the movement of water from the phase in which the solute is.

I *assume that there is free movement of water across* all membranes in organisms in which there is any water on both sides, but that the restriction lies in the changes of volume. Was this what Dr Opie's work was on?

Gaudino No, Dr Opie's work (53) was this: he took pieces of tissue, muscle, liver, skin, and so forth, put them in so-called isosmotic solutions, and measured their increase in weight during the course of time In other experiments, he took similar pieces of tissue, and put them in increasingly concentrated solutions until that increase in weight was prevented He observed that in tissues like liver, kidney, muscle, and others, the osmotic concentration of the liquid in which no change in weight occurred was far above that of the plasma; that is, instead of being 300 milliosmols, it was about 400, 500, or even 600 From the results of this work, the question is raised as to what sort of osmotic equilibrium exists in such tissues

Fremont-Smith Did he take care of postmortem enzymatic breakdown?

Gaudino No, he did not.

ability and permeability, so the fact that a membrane is alive does not mean that the membrane has to do work with respect to the movement of fluid across it It may mean that its life process is maintaining the characteristics that it has, which could conceivably be reproduced with cellophane, although no one has done it

Holbrook Isn't it all a question of oxygen tension?

Gaudino I do not think that anybody has measured oxygen tension inside the cells

Ragan I wonder whether anyone has done this type of thing on tissue culture material? We do not know whether the material, after it is placed in tissue culture, will then have a gradient, as it should have?

Gaudino That is what I am planning to study in the future

Fremont-Smith I believe that tissue cultures do not swell up, when they are bathed in isotonic solution

Gaudino On the assumption that proteins of the plasma may have some action against the gain in weight of tissues immersed in so-called isotonic solutions, I used plasma as the medium for immersion I found that even plasma of the same animal from whom the tissues are obtained does not seem to have an effect in preventing this gain in weight, nor in preventing the interchange of electrolytes

Fremont Smith We can say that the tissues, when taken out and bathed either in Tyrode's, or another solution, are perfectly normal, which I think is what you are trying to say, and yet they swell Or else we have to say that these tissues, which do not swell during life when we know they are normal, become at least to some extent, abnormal when they are put into plasma

Holbrook Dr Gaudino, did you find that in conditions of hypoxia swelling occurs?

Gaudino I do not know whether these tissues are hypoxic Probably they are, because there is no circulation through those pieces of tissues, although we shake them constantly with a rather primitive method At present, we are trying to improve this

Holbrook Why wouldn't there be hypoxia? How could it be avoided?

Zweifach Why must you assume it is hypoxia? After all, you have subjected this tissue to a traumatic experience, it has been excised What proof do you have that these tissues do not restore their equilibrium once they are put into this tissue culture medium? You are assuming that they continue to be abnormal

Gaudino: I am not assuming that hypoxia is the cause of any of the changes In these experiments, I only wish to know why a tissue,

Fremont-Smith. Curiously enough, this does not happen during life.

Gaudino: According to the current way of thinking, plasma is in an active osmotic equilibrium with tissues. *In vitro* cultures have been done in these tissues, and I have found that they are alive, even after one or two hours of plasma immersion.

Alive does not mean that they are in equilibrium, but on the other hand, there is the fact that in two minutes there is scarcely time to change the whole of the osmotic properties of the cells, especially if the cell membrane is considered to be an inert membrane. Theoretically, plasma should be in osmotic equilibrium with the cells.

Angevine: Your objection is a valid one, and Dr. Opie would be the first to agree with it, but on the other hand, that objection could be applied to all work with homogenates, suspensions, and other things

Fremont-Smith. You mean, as far as osmotic pressure work goes?

Angevine· Yes, or permeability

Fremont-Smith Does this suggest that the interstitial materials are grossly hypertonic to the fluid that bathes them? In that case, by some active process of the membranes the cells are prevented from swelling, and then, when they are bathed in the plasma under the circumstances of your experiment, this active process of the membrane is removed, and osmotic equilibrium is allowed to take place. Is that the implication?

Gaudino. I do not know, although it might be so Actually, I do not think there exists an osmotic equilibrium as usually considered Sodium does not enter in amounts equivalent to the loss of potassium. One mEq of sodium is not exchanged for one mEq of potassium *There is a different rate of exchange for each of the ions* potassium leaves the tissues in amounts differing from those of sodium entering, while chloride entrance is independent of sodium. It seems that each *of these three ions is handled by the tissues differently* The important fact is that they are exchanged, and very rapidly The concentration gradients of potassium inside, and of sodium outside, disappear very rapidly

Fremont-Smith All we have to do to correlate this is to assume that in a very short time the cell has been deprived of oxygen and perhaps of glucose, and the character of the *membrane* has been changed It seems to me that there are two elements to a membrane. It can be very much alive, and in no sense inert in the sense that it is alive, and yet its living property may be what *maintains* it with its particular characteristics These include a certain degree of imperme-

the tissue culture results are abnormal? We are arguing fruitlessly now.

Meyer. All I wished to bring out was that we cannot argue from this equivalent

Gaudino. I wish to add to what I mentioned before, that in the case of skin, changes are almost nil In general, there is only a small increase in sodium and chloride, and a small decrease in potassium, but still the significant increase in water is there.

Travell Dr Zweifach showed us a beautiful illustration of what happens to a cell when it is taken out of its environment In this case it was a red blood cell that just moved out of the plasma into the interstitial spaces, it looked the same, but it was of a totally different composition.

Fremont-Smith Irreversibly different

Travell. Yes, and we may assume no hypoxia, or change in tonicity It simply moved into a different environment, without trauma

Angevine The fact that we have started banks for the aorta and for the eye suggests that we can do a great many things with connective tissue They are in an abnormal environment, but they certainly have not been altered as much as we think

Fremont-Smith They live and recover It does not mean that they have not been altered.

Ragan: Does the aorta recover? It is inert, too

Angevine I do not know what you mean by "recover" It is grafted and goes on working, for all practical purposes

Gaudino I think the consensus of opinion among those who have worked with the aorta graft is that it is just a matrix for the endothelium, connective tissue, and perivascular tissue to grow out on gradually and completely replace the grafted piece of aorta

Krog: Yes, that is right

Holbrook It does not maintain viability as such

Krog. No, it is not comparable to a skin graft

Angevine If one puts the aorta in the icebox, the elasticity remains almost unchanged after several days However, although they look just the same, I am sure that there is something altered

Zweifach. It is amazing that in perfused lymph preparations, oxygen content may be cut down to almost zero without destroying the permeability characteristics The endothelial cell will withstand very low oxygen tension, so that oxygen tension *per se* is not the critical factor there.

Ragan: Does glycerin keep the water from crystallizing, when red cells are frozen?

which supposedly is in equilibrium with a certain fluid of the body, does not retain that equilibrium once it is removed from the organism, even though it is still kept in contact with the same fluid It may be that there exists some factor which is removed when tissues are taken out of their normal environment.

Fremont-Smith: I think it is an unjustified assumption to say that because these tissues swell up, when bathed as you or Dr. Opie have bathed them, this means that they were not in equilibrium with the fluid that bathed them in the body. It seems to me that is a rather far-fetched assumption which might be true, but has not been proved by any means

I think that what Dr. Zweifach says is much more to the point that these tissues, when bathed, have been badly traumatized If they are in small pieces, then they have an enormous surface in the total mixture there of injured cells, and if they are in large pieces, they will not reach equilibrium But you say that they are in small pieces

Gaudino: We have studied large pieces, as well as small pieces In the case of large pieces, for instance in one single piece of muscle which was cut lengthwise with reference to the fibres, and only cut in two transversely in the ends, we observed the same changes as with the small pieces of tissue As a matter of fact, the results are very consistent with the same tissue, be it in large or small pieces, the percentage of gain in water per unit weight in function of time is generally identical in different experiments, the same as the percentage of entrance of sodium and chloride and exit of potassium These changes, of course, increase with the passage of time

Fremont-Smith My guess would be that this is a reaction of injury

Mirsky: This brings to mind Greig's work (56) on the red blood corpuscles She showed that cholinesterase activity of the cell determines the passage of potassium, as well as water, in and out of the cell. Her evidence with erythrocytes is rather convincing in support of the concept that the entry of water into the cell is dependent on glycolysis, which in turn determines the rate of cholinesterase activity.

Gaudino: There has been a great deal of work done on the red blood cell

Meyer: Was the plasma heparinized?

Gaudino: Yes

Meyer: Heparin in the concentration used to keep the blood fluid is certainly not a normal component, and is a very active substance in a physicochemical sense

Ragan: Don't you think it would be better to wait and see whether

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Mirsky: Parkes (57) has done a good deal of work with the sperm in that respect Freezing apparently prevents the crystals from forming The glycerin does appear to get in. Then it is quite viable The best index of permeability that I know of is that the sperm will fertilize ova

Fremont-Smith It is a fairly good index, although ova can be fertilized without it Even viability has relativity.

Krog. Can skin be compared with muscle tissue? I was thinking that hypoxia of skin is known to be very difficult to produce, because in the way it obtains oxygen, skin is apparently different from other tissues.

Gaudino You mean that skin is a tissue that normally could be more hypoxic than others?

Krog. Yes, because it seems to be able to take up some oxygen directly from the air

Gaudino I have done the same experiment with skin as with muscle Skin does not seem to change much, as I have mentioned before. Actually, there is practically no change as compared with muscle, liver, or any of the other tissues which show such marked electrolyte changes However, skin also gains water. In all tissues studied, the location of this gain in water is not known. I do not know if it remains localized in the interstitial spaces, or if water also increases inside the cells

Fremont-Smith: You would like to know, particularly when you cut it into small pieces, that the water swelling does not go primarily into the injured cells?

Gaudino That is partly it Actually, most of our work has been done with large single pieces, about a centimeter long The total weight is about one gram. In general, we have only a small surface as compared to the mass, but in spite of that we obtain the same results as with small pieces

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HORMONAL EFFECTS ON CONNECTIVE TISSUES *

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AN IMPORTANT component of the mucinous ground substance of the connective tissue is found in the acid mucopolysaccharides, which are combined in some way with proteins In the skin, hyaluronic acid and chondroitin sulfuric acid have been demonstrated Both substances stain metachromatically with toluidine blue According to Meyer and Rapport (1), however, only the hyaluronic acid is suspectible to hyaluronidase.

A conspicuous change observed in connective tissue under the influence of cortisone, is the subsidence of the hyaluronidase-sensitive metachromatic substance (2) This change has been observed even in skin, and may be due to an alteration in the amount and chemistry of hyaluronic acid

Since hyaluronic acid is a physiologically active substance, these changes must be essential factors in the effect of cortisone on a large number of disorders in which a pathological increase in hyaluronic acid is a predominant feature, and of pathogenetic significance The action upon the ground substance, I have tried to elucidate by more indirect methods

Spreading experiments during cortisone treatment showed an inhibition of the action of hyaluronidase in the dermis, and an increase in the direct spread of a dyed or radioactive indicator in the tissue without hyaluronidase My interpretation of these findings is that during cortisone treatment, the injected enzyme finds less and altered substrate to act upon, and thus the spreading power is reduced (3)

The effect of cortisone upon the connective tissue ground substance, and upon hyaluronic acid, cannot be considered without also studying the cells which form it Some previous investigations (4, 5) have indicated that the mast cells, under hormonal influence, evidently secrete mesenchymal mucopolysaccharide hyaluronic acid, perhaps re-

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Since hyaluronic acid is a physiologically active substance, these changes must be essential factors in the effect of cortisone on a large number of disorders in which a pathological increase in hyaluronic acid is a predominant feature, and of pathogenetic significance The action upon the ground substance, I have tried to elucidate by more indirect methods

Spreading experiments during cortisone treatment showed an inhibition of the action of hyaluronidase in the dermis, and an increase in the direct spread of a dyed or radioactive indicator in the tissue without hyaluronidase My interpretation of these findings is that during cortisone treatment, the injected enzyme finds less and altered substrate to act upon, and thus the spreading power is reduced (3)

The effect of cortisone upon the connective tissue ground substance, and upon hyaluronic acid, cannot be considered without also studying the cells which form it Some previous investigations (4, 5) have indicated that the mast cells, under hormonal influence, evidently secrete mesenchymal mucopolysaccharide hyaluronic acid, perhaps re-

* This work was supported by Eli Lilly and Company, Indianapolis, Indiana, The National Danish Association against Rheumatic Diseases, and The National Danish Anti Cancer League

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HORMONAL EFFECTS ON CONNECTIVE TISSUES *

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sembling heparin in the preliminary stage. I wished to determine whether cortisone acted upon the ground substance, or whether its point of attack was the actual site of its formation.

Following administration of ACTH or cortisone in high doses, human beings, rabbits, guinea pigs, and mice, show a considerably reduced number of mast cells in the connective tissue, and those which are visible exhibit marked morphological changes (2, 6, 7). There are invariably some cells which do not appear to differ from normal, and they are nearly always in a perivascular situation. While the granules are normally of almost uniform size, and are evenly and uniformly distributed in the cytoplasm, the mast cells in tissue subjected to the action of cortisone are more or less degranulated. The granules are accumulated in conglomerates of varying size. A number of them show cytoplasmic vacuoles, too The partitions which separate these vacuoles appear to consist of a homogeneous or partly granular substance, staining partly orthochromatically, and partly metachromatically, with toluidine blue The contours and shape of the cells are irregular and even bizarre, they vary in size, but are mostly small

Figure 26 (opposite p. 146) shows a preparation of skin connective tissue, stained with toluidine blue. We observe two mast cells which are saturated with granules and metachromatically stained, and some fibroblasts. In Figure 27 (opposite p. 146) we see two mast cells of connective tissue under cortisone influence; also the vacuoles in the cytoplasm With some difficulty a slight metachromasia, left here and there, may be observed

Porter. The other cells in the preparation seem to have changed also, is that right?

Asboe-Hansen. I do not think that these very small fields can show that

Porter. But they are fewer in number?

Asboe-Hansen: I doubt that they are fewer in number, if we examine the whole preparation.

Figure 28 presents a normal mast cell by high magnification We see the central nucleus with a little notch, and the granules evenly distributed over the cytoplasm. Figure 29, shows a mast cell from connective tissue under cortisone influence, showing vacuolation and conglomeration of the granules. In Figure 30 we see another mast cell from a preparation of human skin subjected to an equal amount of cortisone. The granules are conglomerated in smaller and greater aggregates.

Holbrook: Dr. Asboe-Hansen, is the cortisone applied locally to the tissue, or given systemically?

Asboe-Hansen· It is given systemically. Figure 31 shows a third variation of mast cells with vacuoles, conglomerated granules, and some normal granules. We may find all these variations of cells in one prep-



FIGURE 28



FIGURE 29



FIGURE 30



FIGURE 31

FIGURE 28 Normal mast cell Granules evenly distributed throughout the cytoplasm Magnification approximately 3,000 x

FIGURE 29 Mast cell from human skin influenced by cortisone Vacuolation and conglomeration of granules Magnification approximately 3,000 x Reprinted, by permission from Asboe Hansen, G The mast cell Cortisone action on connective tissue *Proc Soc Exper Biol & Med* 80, 677 (1952)

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677 (1952)

FIGURE 31 Mast cell from human skin influenced by cortisone Granules irregularly distributed partly in the periphery, partly in lumps around the central nucleus, and also around the vacuoles Magnification approximately 3,000 x Reprinted, by permission, from Asboe Hansen, G The mast cell Cortisone action on connective tissue *Proc Soc Exper Biol & Med* 80, 677 (1952)

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FIGURE 30 Mast cell from human skin influenced by cortisone Granules distributed in major and minor clusters and lumps staining orthochromatically or metachromatically Magnification approximately 3 000 x Reprinted, by permission from Asboe Hansen, G The mast cell Cortisone action on connective tissue *Proc Soc Exper Biol & Med* 80 *677 (1952)*

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aration under cortisone therapy. All the preparations were prepared with 5 per cent lead subacetate solution.

If one has studied mast cells, and found all transitions between complete granulation and an almost entire absence of any granulation, one is convinced that the explanation of why tissue subjected to the action of cortisone exhibits fewer mast cells is primarily that many of them have lost all their granules, and thereby their histological characteristics I tried to investigate these problems by electron microscopy, in collaboration with Dr F Carlsen, of the Laboratory of Biophysics, University of Copenhagen These examinations reveal marked changes in the mast cells, and indicate that all the mast cells are changed in cortisone-influenced tissue



FIGURE 32 Electron micrograph of a normal mast cell. Magnification approximately 14,000 x

To study these phenomena in the electron microscope, I chose fixation with basic lead acetate I selected this method for histochemical purposes, well knowing that it is not a good fixation method for electron microscopy I also used one per cent osmic acid by the method of Palade, and freeze-drying After fixation, the specimens were embedded in plastic and cut into ultrathin sections Mast cells from normal, untreated connective tissue appeared with or without cell membrane The nucleus was of marked and uniform density, at least in some preparations easily distinguishable from the fibroblast nucleus, the chromatin density of which was distinctly less marked and less uniform The granules, spherical or slightly oval, and very dense, were distributed diffusely in the cytoplasm All seemed to be of practically the same size, that is, 0 3 micra Any variations in the granule size were minimal

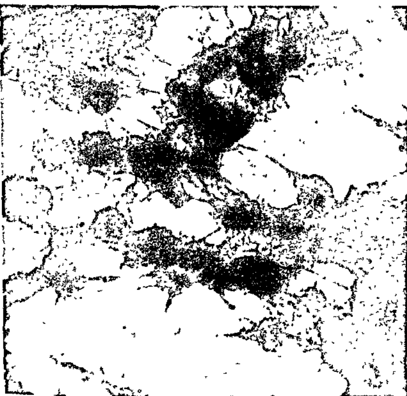


FIGURE 33 Electron micrograph of normal mast cell granules Magnification approximately 50,000 x

mucopolysaccharides, or polyestersulfates, incorporate radioactive sulfur, which is administered as labeled sulfate. Wherever it is present in a histological section, it causes the blackening of a sensitive emulsion, when placed in direct contact with the preparation

For the autoradiographic studies of mast cells, and the effect of cortisone upon these cells, I selected connective tissue containing great quantities of large mast cells, which had recently appeared in the tissue owing to experimental provocation There were various possibilities, but I chose mouse skin, painted once with the carcinogenic hydrocarbon 9, 10-dimethyl- 1, 2-benzanthracene. At a certain stage, the connective tissue of experimental skin tumors, induced by painting with carcinogenic hydrocarbon, contains myriads of large and amply granulated mast cells

After papillomas had been induced in a series of mice, half of them were treated with cortisone in a dosage of 2 5 mg., administered daily for 13 days The untreated tumor-bearing mice, as well as the cortisone-treated ones, were injected intraperitoneally with S^{35}. On the eleventh day we gave them 8 microcurie per gram of body weight, with 0 1 mg of sodium sulfate as carrier in 0 2 ml of sterile water. Forty-eight hours after the injection, the mice were killed

Specimens of the tumors, as well as of normal skin, were fixed in 70 per cent alcohol, 10 per cent formalin, and 4 per cent lead subacetate solution, or freeze-dried The tissue was then embedded in paraffin, and cut into sections about 7 micra thick The sections were deparaffinized and dried, and autoradiographs were made by the stripping-film technique of Pelc. We also used the floating method of Evans to some extent

The films were exposed for 17 days The unstained preparations were studied and photographed by light-field, phase-contrast, and dark-field microscopy, the microscope being focused partly on the film and partly on the tissue section Thereafter, the tissue section was stained through the film with a 0 5 per cent solution of toluidine blue for 15 minutes The results, after 17 days' exposure, were as follows:

In preparations from untreated mice, the film showed marked blackening over the mast cells in the connective tissue. Moreover, there was a diffuse, less intense blackening over the surrounding connective tissue Autoradiographs of unpainted skin showed practically no blackening. Some mast cells, particularly in the deeper layers, for instance in the muscles, did not appear to take up any S^{35}, even though they were granulated and metachromatically stained.

In the tumor connective tissue from a cortisone-treated mouse, the difference in the intensity of blackening was considerably equalized; that is, there was considerably less blackening over the mast cells. This means that they contained considerably less radioactive sulfur than mast cells in untreated connective tissue. In the ground substance, the content was unchanged or slightly reduced. Monitoring with a Geiger counter showed marked accumulation of radioactive sulfur over the tumors of untreated as well as cortisone-treated mice, and somewhat less over the latter.

If we focus the microscope on the tissue section, we may see the metachromatic mast cells here and there. [illegible]
for example the Eva [illegible]
we see tracks causec [illegible]
are not very distinct.

Gaudino: What was the thickness of the emulsion?

Asboe-Hansen: It was around 25 micra, which is rather thick, the stripping film was only a few micra.

In Figure 36 (opposite p. 146), the microscope is focused on the film, we see a very marked accumulation of radioactive sulfur, the blackening being produced by the one mast cell, and to a slighter degree by the other.

Figure 37 shows a histological preparation of a papilloma of a mouse, painted with the carcinogenic hydrocarbon, we see the huge amounts of mast cells in the connective tissue of these tumors.

Figure 38 is an autoradiograph of a mouse papilloma. The microscope was focused on the film, and the photograph was taken by light-field microscopy. As we see, the epidermis gives no blackening. Over the connective tissue, on the other hand, we find blackening of the film, intensely over the mast cells, and more slightly over the connective tissue ground substance. There is a rather great difference between the uptake of sulfur in the mast cells and in the ground substance.

In normal, unpainted skin we may see hairs at the top. In painted skin no hairs can be seen because the painting causes depilation. There is practically no blackening of the connective tissue. We may see a mast cell here and there, but there is practically no radioactive sulfur in these tissues.

Figure 39 is a high magnification by light-field, showing the mast cells, and giving us an idea of the resolution.

Figure 40 shows a stained preparation, we observe a perivascular area, some fibroblasts around the vessel, and two mast cells which give strong blackening of the stripping film.



FIGURE 37 Mouseskin, 20 days after painting with 9, 10-dimethyl 1, 2-benzanthracene Enormous quantities of mast cells in the connective tissue



FIGURE 38 S^{35} autoradiograph of experimental skin tumor Unstained preparation Light field microscopy No blackening over the hyperplastic epithelium Intense blackening over the mast cells, less intense over the connective tissue ground substance Magnification approximately 600 x

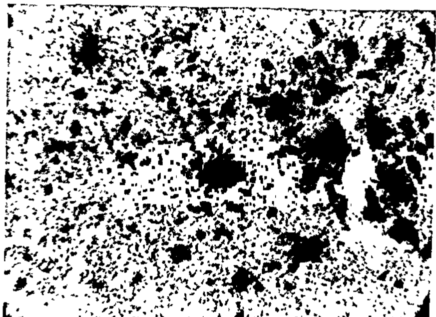


FIGURE 39. [illegible]

Porter: This is control material?

Asboe-Hansen: Yes.

Figure 41 is an autoradiograph of a tumor. I should like to point out the difference between the strong blackening produced by the mast cells, and the slight blackening over the ground substance. Figure 42 shows a tumor after cortisone therapy. We see that the difference is very much equalized. The mast cells can still be discerned, but the uptake of sulfur in these cells is much slighter than before cortisone therapy

Meyer: Isn't there a greater diffusion?

Asboe-Hansen: It [illegible] so, [illegible] is not. The autoradiographs were [illegible] been fixed in formalin, alcohol, [illegible] itoring with a Geiger [illegible] Figure 41 there is [illegible] ration.

Meyer: But how [illegible]

Asboe-Hansen: Not [illegible]

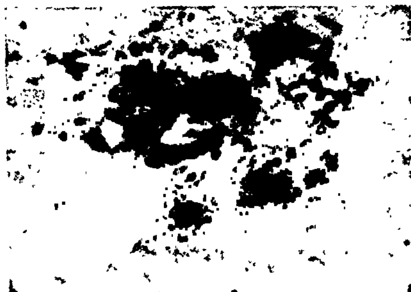


FIGURE 40 S^{35} autoradiograph. Section stained with toluidine blue. Two perivascular mast cells give intense blackening of the film. Magnification approximately 2,300 x. Reprinted, by permission, from Asboe-Hansen, G. Autoradiography of mast cells in experimental skin tumors of mice injected with radioactive sulfur (S^{35}). *Cancer Res 13* 587 *(1953)*

the first preparation may play a role. There is not a very great difference if the connective tissue is taken as a whole. The relative difference between the mast cells and the ground substance is equalized in all these preparations.

Gaudino: Do you have a light-field autoradiograph of the cortisone-treated mice?

Asboe-Hansen. Yes, but I show these dark-fields because the visual contrast is higher with photography by dark-field, there is a quantitative difference which cannot be obtained with the light-field microscopy.

Meyer: This is not sensitive to hyaluronidase?

Asboe-Hansen: I have not tried it in these tumors, but I should suppose it was not.

Holbrook. Will the mast cells in the area of the tumor concentrate the sulfur more than mast cells that are somewhere else?

Asboe-Hansen. Yes, I think so. But in unpainted skin, one may find mast cells giving rather intense blackening, too. I think the difference is not very marked, but I have just chosen these tumors as my study material.



FIGURE 41 Autoradiograph of histological section through experimental skin tumor



GURE 42 S^{35} autoradiograph Cortisone-treated mouse Dark field microscope fo-
sed on the film Magnification approximately 600 x Radioactive sulfur content of the
ast cells lower; that of the surrounding connective tissue relatively high The difference
onsiderably equalized Reprinted, by permission from Asboe-Hansen, G Autoradio-
aphic evidence of cortisone action on mast cells in experimental skin tumors *Cancer Res*
4, 94 (1954).

Holbrook: Because there were so many mast cells?

Asboe-Hansen: Yes.

Meyer: Do you know the total sulfate and radioactivity, after isolation of both organic and inorganic sulfate from a given weight of tissue?

Asboe-Hansen: No, we have not measured it chemically.

Meyer: That might be interesting, especially in view of the diffuse staining The possibility is that we measure not only carbohydrate sulfate, but also other organic sulfates, such as phenolic sulfates

Gaudino. Presumably all types of sulfate are being measured, depending on the rate of exchangeability. The time (48 hours) is about the same in all cases, therefore S^{35} probably had been able to diffuse completely

Meyer: I believe in 24 to 48 hours the maximum of the injected sulfate is fixed, as shown by Dziewiatkowski (10), and by Bostrom (11).

In normal skin, or in tumor, can one influence the total sulfate uptake, as measured in the degree by blackening, by the quantity of sulfate injected? From the work of Bray and Thorpe (12), there appears to be a deficiency of sulfate for detoxification purposes These authors showed that the normal conjugation of phenolic compounds, such as β-glucuronides, is displaced by conjugation with sulfate if the available concentration of sulfate is raised by injection I wonder whether one could push the synthesis or the exchange of sulfate in the mast cells, for example, by injection of radioactive sulfate in increasing total sulfate concentration.

Asboe-Hansen No, I know of no such experiment.

Gaudino: How much carrier sulfate did you use?

Asboe-Hansen We used 0 1 mg

Meyer: It is a very low concentration.

Sinex. What would be the daily sulfur intake, in amino acid, in an animal like that?

Meyer: The amount of sulfur available from amino acid must be extremely small

Asboe-Hansen: During the same experiment, we studied the content of radioactive sulfur in the hair papillae and follicles The papillae and the connective tissue surrounding the hair follicles contain rather ample quantities of a metachromatic substance. Autoradiography also shows an accumulation of radioactive sulfur in these sites, and even in the keratogenous layer of the hair follicle The epithelia of this prekeratinous zone are known to contain sulfur in SH and SS compounds.

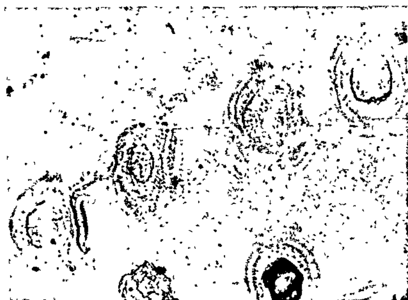


FIGURE 43 S^{35} autoradiograph Light field microscopy Four hair follicles Distinct blackening of the film over the keratogenous zones

Figure 43 shows light-field autoradiographs of hair follicles We note the rings of dots over the keratogenous zones Figure 44 shows one hair bulb with a transversely cut papilla in the center, giving strong blackening of the film This is dark-field microscopy

Gaudino I suppose this is stripping film?

Asboe-Hansen: Yes, that is right

Meyer. Is it possible that this might be inorganic ionizable sulfate, let us say bound as protein sulfate?

Asboe-Hansen· But could it be present in such large quantities as shown here?

Meyer. It would have to show up histologically, I suppose. The amount is relatively rather small here I do not know anything about the characteristics of the protein in this area, or the material, whatever it is

Asboe-Hansen. One can obtain strong metachromatic staining of the tissues surrounding the hair follicles But I have no explanation of the blackening over the prekeratinous zones of the follicles after injection of labeled sulfate

Sinex: We cannot go backwards from sulfate to sulfhydryl, can we?

Asboe-Hansen: That is the problem; if cystine is isolated, after injection of labeled sulfate, there is no radioactive sulfur within the cystine. The greater part of the sulfur within these zones is thought to be in cystine, or organic SH, is it not?



FIGURE 44 S^{35}-autoradiograph Dark field microscopy Intense blackening over the hair papilla protruding from the connective tissue into the hair bulb

Sinex. Yes Have you isolated the cystine, Professor Asboe-Hansen? I believe Dziewiatkowski (13) is doing something like that

Asboe-Hansen: No, but Tarver and Schmidt (14) have. While the hair follicles from untreated mice showed unmistakable local accumulations at the site of the papilla and in the keratogenous zones, the cortisone preparations showed some blackening over the papillae. However, we never saw strong accumulations, or rings in the cortisone preparations.

Since mast cells and mucopolysaccharides make up a conspicuous element of the connective tissue in skin carcinomas and precancerous papillomas, I was interested in investigating the effect of cortisone upon tumor growth, as such, with an eye to the mast cells of course. This work was done in collaboration with Dr. Engelbreth-Holm, of the University Institute of Pathological Anatomy in Copenhagen (15). We performed these experiments by injecting cortisone into albino St/Eh mice, which were painted once with the carcinogenic hydrocarbon, 9, 10-dimethyl-1,2-benzanthracene. Intraperitoneal injections of 0.2 mg. daily for seven weeks, beginning on the day prior to painting, proved to inhibit the development of tumors and considerably increase their latent period. Under the influence of cortisone, the mast cells showed the same morphological changes observed in normal connective tissue. These mice, which developed tumors despite the administration of cortisone, also exhibited numerous mast cells.

Table II shows the results of one of the experiments with tumor growth. Table III gives the sex difference. The role of the mast cells in tumor formation and growth is unknown. Certain findings, especially those of Cramer and Simpson (16), appear to indicate that they make up a sort of barrier against tumor growth.

TABLE II

Number of skin tumors developing after one painting with 9,10 Dimethyl-1,2-Benzanthracene in female St/Eh mice. The experimental animals received a daily dose of 0.2 mg. Cortisone throughout the experiment, starting on the day before the painting.

	Cortisone treated	Controls
Incidence of Papillomas	17/34 (50%)[1]	27/30 (90%)[2]
Average Latent Period in Days ± Standard Error	35.5 ± 2.09	25.7 ± 0.84
Average Body Weight in Grammes ± Standard Error	20.5 ± 0.427	21.4 ± 0.502

[1] Among the 17 tumour bearing animals 2 carcinomas and 2 leukaemias were found.
[2] Among the 27 tumour bearing animals 2 carcinomas and 4 leukaemias were found.

Reprinted by permission from Engelbreth-Holm, J. and Asboe-Hansen, G.: Effect of cortisone on skin carcinogenesis in mice. *Acta path. et microbiol. scandinav.* 32, 560 (1953).

Porter. Is this tumor growth or tumor genesis?

Asboe-Hansen. It is tumor growth and tumor genesis. Cramer and Simpson found a very high incidence of mast cells in mice which were resistant to tumor development, and they found a decrease in the number of these mast cells when the tumors went from a precancerous stage to the carcinomatous stage.

TABLE III

Number of papillomas after one painting with 9,10-Dimethyl-1,2-Benzanthracene in St/Eh mice. The experimental animals received a daily dose of 0 2 mg. Cortisone throughout the experiment, starting on the day before the painting

	Cortisone-treated		Controls	
	Males	Females	Males	Females
Incidence of Papillomas	3/28 10 71%	13/27 48 15%	9/26 34 62%	20/24 83 33%
Average Latent Period in Days ± Standard Error	(44 3)	37 77±3 35	41 8±5 11	31 15±1 79

Reprinted by permission, from Engelbreth Holm, J., and Asboe-Hansen, G Effect of cortisone on skin carcinogenesis in mice *Acta path et microbiol scandinav* 32, 560 (1953)

Quite recently, I continued the cortisone experiment in collaboration with Dr. Zachariae (17) of my laboratory. We injected 1 mg. of hydrocortisone into the connective tissue of fully developed tumors of the same type as those just described. Control mice were injected with physiological saline solution In the course of seven weeks, the tumors subsided in 83.1 per cent of the mice treated with hydrocortisone, and in 36 2 per cent of those injected with saline solution At the end of another seven weeks, the number of tumors was the same (Table IV).

TABLE IV

	When treatment was started	Number of mice with tumors								Tumors disappeared at end of 8 weeks
		1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	
Control	58	58	56	53	46	44	38	37	37	36 2%
Hydrocortisone	65	62	51	27	22	16	12	11	11	83 1%

Table V gives the sex differences In the female control group, 6 per cent of the tumors disappeared, 65 per cent disappeared after hydrocortisone In the male group, 48 per cent of the tumors disappeared in the control group, and 95 per cent in the hydrocortisone-treated group

Travell: How does the frequency of subsidence of the tumor without any procedure compare with that observed after isotonic saline solution is injected into it?

Asboe-Hansen: It is just the same.

Travell: Is that the natural course?

TABLE V

	When treatment was started	Number of mice with tumors								Tumors disappeared at end of 8 weeks
		1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	
Control ♀	16	15	15	15	15	15	15	15	15	6%
Control ♂	42	43	41	38	31	29	23	22	22	48%
Hydrocortisone ♀	23	22	21	15	13	12	9	9	9	65%
Hydrocortisone ♂	42	40	30	12	9	4	3	2	2	95%

Reprinted by permission from Zachariae L, and Asboe-Hansen G Regression of experimental skin tumors in mice following local injections of 17 hydroxycorticosterone 21-acetate *Cancer Res* **14**, 488 (1954)

Asboe-Hansen. Yes, it is known to be just the same

Travell: So the saline itself had no influence?

Asboe-Hansen None at all I think the effect of the adrenocortical steroids on the connective tissue mucinous system must be an important factor in these conditions we have been discussing The influence of cortical steroids on infection, on the process of bacterial or allergic inflammation, is no doubt to some extent due to the alteration of the connective-tissue ground substance, the very soil of the inflammatory process It affects the course and the power of the bacteria and toxins. The spread of bacteria and toxins in the tissue is influenced, but so is the hyaluronidase effect of the bacteria It is difficult to elucidate what will promote, and what will in the end inhibit, an inflammatory process

Wound healing is affected by cortisone, as demonstrated by Ragan, Howes, Plotz, Meyer, and Blunt, in 1949 (18) Normal wound healing, that is, the new formation of connective tissue, presupposes the presence of a ground substance I imagine that the perivascular cells of a wound produce the mucopolysaccharides of the ground substance. Proteins are supplied by the blood plasma and exuded fluids This gives the conditions for a formation of a normal mucinous ground substance. Other cells move from the vessels and blood stream, fibrils are laid down, new vessels arise, and so on A strong action of cortisone, however, will inhibit the production of mucopolysaccharide, because cortisone influences the mast cells, the supposed site of its formation Exudation is also inhibited Mucopolysaccharides apparently play an essential role in collagen fibrillogenesis. The fibroblasts, which are concerned with the formation of collagenous fibrils, are also inhibited Consequently, the entire process of the formation of new connective tissue is inhibited

Drs. Lis Zachariae and Erik Moltke (19) have shown that local application of hydrocortisone entails considerable morphological alteration of the numerous mast cells present in wounds of rabbit skin. In addition Dr. Zachariae (20) has found the formation of experimental peritoneal adhesions to be inhibited by a depot of hydrocortisone in the peritoneal cavity.

I studied the local effect of hydrocortisone on keloids. The keloids were weakened and flattened, the metachromasia subsided, and the mast cells were found to be degranulated and vacuolated.

Figure 45 (opposite p 146) shows a keloid in human skin. We see the strong metachromasia in the deeper layers; in fresher keloids this is very intense We see the numerous mast cells within these areas, but not in the superficial layers This is a "normal" keloid

In Figure 46 (opposite p 146) we see that ten days after one injection of hydrocortisone, we have a preparation from which practically all of the metachromasia has disappeared. We still find some mast cells scattered over the preparation, but they are fewer, and are morphologically altered

By high magnification of one mast cell one may see large vacuoles Some of the granules are orthochromatic and some are metachromatic The ground substance is faintly metachromatic. These are changes similar to those found in the rabbit and in humans who are treated systemically with cortisone, and are observed after local hydrocortisone therapy

All our findings indicate that cortisone and hydrocortisone exert an inhibitory effect on the new formation of connective tissue The influence of the adrenal steroids on the mast cells in the connective tissue may be a factor of essential importance for this effect, and thus also for the effect on connective tissue disorders Joints consist of mesenchymal tissue, and in rheumatoid arthritis, for example, there is an accumulation of mast cells in the synovial connective tissue, and there are great quantities of mast cells even in normal synovial membrane, which are more or less granulated. In the eyes, we find rather numerous mast cells in the iris and ciliary body, and in iridocyclitis and glaucoma their number is enormously increased. In several dermatological diseases, such as pemphigus, acute disseminated lupus erythematosus, fresh cases of scleroderma, urticaria, and many others, there may be parallel findings

In preparations of normal synovial membrane, stained with toluidine blue, we find rather large accumulations along the lining of the joint cavity (Figure 47, opposite p 146).

In the deeper layers, there are heavily granulated mast cells. Among

the lining cells the mast cells are faintly stained This preparation is differentiated with acetic acid. If we do not differentiate, we shall find rather strong metachromasia of the ground substance in the superficial layers, but fainter in the deeper layers. These mast cells are not visible because they are slightly metachromatic, and with little or no granulation.

We find mast cells within and along the lining of the synovial cavity. This has been denied by several workers, but there is no doubt that quantities of them may be there. However, many of these superficial cells are degranulated, and therefore may be demonstrated only if ideal methods are used, primarily fixing with basic lead-acetate, or freeze-drying.

Angevine. Are these found in every synovial membrane?

Asboe-Hansen: Yes, but in some areas they are more numerous than in others. In a normal eye there are a large number of mast cells in the iris, and in the ciliary body

Meyer: Did you investigate the mast cells after an $S^{35}O_4$ injection?

Asboe-Hansen: No, I did not do that, but I think studies of that are going on now

Meyer: Have you examined synovial linings?

Asboe-Hansen: No, we have not, but I think such investigations are in preparation Now for years, it has been known that thyroid insufficiency produces a condition called myxedema, meaning mucinous edema, and named after the accumulations of mucin in the dermal connective tissue of such patients The mucinous substance consists predominantly of mucopolysaccharides combined with protein, and these mucopolysaccharides are hyaluronic acid and chondroitin sulfuric acid According to Watson and Pearce (21), this also applies to localized myxedema, which has been observed in patients with hyperthyroidism, usually after partial thyroidectomy, and which is almost invariably accompanied by exophthalmos In both conditions there is an enormous accumulation of mast cells, in addition of mucopolysaccharides

Figure 48 (opposite p 146) shows a skin preparation of a patient with localized myxedema in which there was no increase of the metabolic rate, and with extreme exophthalmos This preparation is stained by toluidine blue, and we see an extraordinarily strong metachromasia in the dermis, because of the high content of mucopolysaccharides There are very numerous mast cells in these areas We can just discern the strongly metachromatic cytoplasm, but it may be difficult because of the intense metachromasia of the ground substance.

Apparently these connective tissue changes are not due to degeneration, but to cellular activity. The state is reversible, regulated by internal secretion The myxedema yields to treatment with thyroxin During such treatment, the mast cells also change from being large and well-granulated, to small and degranulated cells. In dermal connective tissue from thyrotoxic subjects, the ground substance stains very faintly with toluidine blue The only place where slight metachromasia may occur is in the papillary layer. Only a few mast cells are demonstrable, and practically all of them of a perivascular habitat.

Myxedema is nearly always accompanied by some, and often by marked, accumulation of water in the tissues, perhaps because of the water-binding property of hyaluronic acid (22). The bundles of collagen fibrils are often, particularly in severe cases, basophilic; they swell and burst apart in an extremely irregular and loose fashion. Even deep in the submucosa, the mucin makes up a prominent part of the tissue, and there is often a considerable loss of adipose tissue. Some keratosis is nearly always seen in the epithelium. These changes are present in varying degrees in cases of hypothyroidism, and also where

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FIGURE 26 *Connective tissue from human skin, two metachromatic granulated mast cells Staining toluidine blue, one-half per cent aqueous solution*

FIGURE 27 *Dermal connective tissue of man under the influence of cortisone Two mast cells with vacuolated cytoplasm Slight metachromasia is left, granules not discernible*

FIGURE 36 *Autoradiograph, microscope focused on the film Blackening over two mast cells, identified by their metachromatic granules*

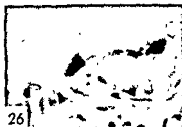
FIGURE 45 Keloid in human skin Staining toluidine blue Intense metachromasia of the *ground substance*, numerous mast cells

FIGURE 46 Keloid in human skin ten days *after one* local injection of hydrocortisone Most of the *metachromasia* has subsided, the mast cells are *degranulated* and vacuolated

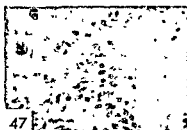
FIGURE 47. Synovial membrane Staining, toluidine blue, differentiated with acetic acid, *two* per cent Intensely *granulated* mast cells in the deeper layers, faintly granulated or *nongranulated* mast cells in the lining of *the* synovial cavity Reprinted, by permission, from Asboe-Hansen, G *On the Mucinous Substances of Connective Tissue* Copenhagen, Rosenkilde & Bagger, 1951

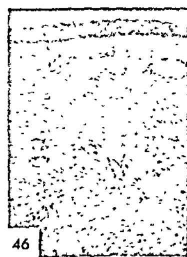
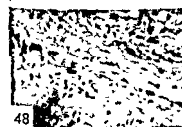
FIGURE 48 Dermal connective tissue from localized pretibial myxedema. Staining toluidine blue Ground substance *strongly metachromatic* Numerous metachromatic *mast* cells

FIGURE 49 Transversely cut skeletal muscle (quadriceps *femoris*) from a patient with malignant exophthalmos Staining toluidine blue. Intrasarcolemmic accumulations of a metachromatic substance ('*half moons*')


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the myxedema is invisible In the synovial fluid, the amount of hyaluronic acid may be considerably increased (22)

There is much to indicate that myxedema, induced by thyroid disorder, is due to a preponderance of thyrotropic hormone in relation to the hormone of the thyroid gland A complete or partial loss of thyroid function will give rise to a disturbance resulting in a preponderance of the pituitary hormone In myxedema, several workers have found an increased content of thyrotropin in the blood plasma, and others have found an increased excretion in the urine. Patients with thyrotoxicosis may also exhibit an excretion of thyrotropin, but in an inactivated form. Even *in vitro* sections of the thyroid gland are capable of inactivating the thyrotropic hormone, as shown by Rawson and his associates (23). Generally speaking, the thyrotropic pituitary hormone is considered to be an essential pathogenetic factor in localized myxedema, and in malignant progressive exophthalmos In these conditions, increased quantities of thyrotropin have been found in the serum

The changes present in the connective tissue in cases of myxedema, primarily the accumulation of mucopolysaccharides, correspond rather closely to the changes demonstrable in the connective tissue of guinea pigs treated with thyrotropic hormone

In collaboration with Dr Kurt Iversen (24, 25), I performed a number of animal experiments on the effect of thyrotropin on connective tissue These experiments again led to investigations of human subjects with pituitary-thyroid disorders Guinea pigs, intact as well as thyroidectomized, and injected with thyrotropic anterior pituitary extract, as early as three to twenty-four hours after the injection, show a mobilization of fat from the normal depots, and a transport, by way of the blood stream, to the skeletal and heart muscles, liver, and kidneys During this period, there will be quite marked lipemia.

We studied the adipose tissue in the retrobulbar, axillary, perirenal, and peritesticular depots During the days immediately following the first injection, we saw the intracellular droplets of fat becoming smaller and dividing, while the septa between the cells, and between groups of cells, became broader, and the tissue exhibited infiltration by mast cells, lymphocytes, and histiocytes Gradually, the adipose tissue was replaced by a mucinous, metachromatic, substance, susceptible to the action of hyaluronidase, and behaving like a mucopolysaccharide of the hyaluronic acid type Exophthalmos appeared three to thirteen days after the first injection. Knowing that hyaluronic acid has a marked capacity to bind water, we imagined that the tissue change which had taken place, that is, the accumulation of a water-binding

polysaccharide instead of fat, was the real cause of the bulbar protrusion.

At the same time, Ludwig, Boas, and Soffer (26) performed practically the same experiment, even on the same theoretical basis They found increased quantities of hexosamine after hydrolysis of retrobulbar tissue Hexosamine is an essential component of mucopolysaccharides The fat-mobilizing, and exophthalmogenic effect, therefore appear to go hand in hand, one being a consequence of the other.

The fat-mobilizing factor is inhibited by thyroxin. In an experiment, we induced thyrotoxicosis in guinea pigs with thyroxin, increasing the basal metabolic rate (BMR) by about 30 per cent, and thereafter the animals were treated with thyrotropin Afterward we found no accumulation of fat in the liver, kidneys, or muscles On the other hand, the fat mobilization caused by a thyrotropic pituitary extract was uninhibited in animals whose BMR was raised 30 per cent by intramuscular injections of sulfuric oil.

In addition to its fat-mobilizing and exophthalmogenic effect, the pituitary extract possesses a thyroid-stimulating effect It is capable of increasing the cell volume, the parenchyma of the thyroid gland

It had previously been shown by Smelser (27) and others that experimental thyrotropic exophthalmos, as well as thyroid stimulation by an anterior pituitary extract, was inhibited by thyroxin

Our preparation (Th 10) of thyrotropin was made by Parke, Davis and Company, the Armour Laboratories, and by the National Institute of Medical Research in London

Sinex It is interesting that thyrotropin is reported to be 2.5 per cent hexosamine. It that correct?

Asboe-Hansen: Yes, it is 2 5 per cent glucosamine. All these preparations have proved to possess a fat-mobilizing, an exophthalmogenic, and a thyroid-stimulating effect The hormone was produced with a view to obtaining as pure a thyrotropin preparation as possible (Five mg of the preparation, Th 10, contain 0.05 i u of ACTH, but no demonstrable growth factor The dose given to the guinea pigs was 1 mg) I am not prepared to say whether we are dealing with three hormones, or with three partial effects of the same hormone But apparently, all these factors are specifically inhibited by thyroxin.

Jefferies (28), and Dobyns and Steelman (29), contend that by chemical action they can separate the thyroid-stimulating from the exophthalmogenic effect These are important contributions to the discussion, but a consideration of two separate partial effects by chemical action should not blind us to the fact that we are dealing with only one hormone. On the other hand, the exophthalmogenic principle seems to predominate in malignant exophthalmos, in the fat-mobiliz-

ing process in myxedema, and in the thyroid-stimulating factor in thyrotoxicosis.

After the animal experiments, we began studying human subjects with pituitary-thyroid diseases, such as malignant exophthalmos, thyrotoxicosis, myxedema, and acromegaly Our purpose at the beginning was to look for fat in the muscles, in the retrobulbar tissues from patients with exophthalmos, and so forth, but it proved difficult to get preparations fixed in lead subacetate

In 1951, we began obtaining biopsy specimens from muscles, primarily the quadriceps femoris and the biceps brachii, and found practically no fat in them One-half of each tissue specimen was fixed in formalin, and frozen sections were studied after Sudan staining, but we found little or no fat The other half was fixed in 4 per cent lead subacetate solution In the latter preparations, we found muscular changes which predominated in the microscopic picture (30)

Following staining with toluidine blue, we found metachromatic, homogeneous masses within the sarcolemma, but outside the muscular substance proper We had not expected that We knew that in myxedema and in malignant exophthalmos we should find accumulations of mucinous substances interstitially This is all over the connective tissue system, for example in the skin and between the muscle fibers But here we found the substance inside the sarcolemma In transverse sections, it appeared in semilunar masses, in longitudinal sections, it was spindle-shaped This substance was susceptible to hyaluronidase, its polysaccharide content was further confirmed by the McManus-Hotchkiss staining method, as these half-moons stained an intense red after two minutes in Schiff's solution In Hale's method, in which iron is bound to the acid polysaccharides in the tissues and then demonstrated *in loco* with potassium ferrocyanide, the masses stained Prussian blue Stained with hematoxylin-eosin, they were practically invisible, and by the van Gieson-Hansen method they stained yellow, but not very outstandingly Formalin-fixed preparations showed no such masses Upon very close inspection, however, their presence was indicated by empty spaces within a loose-fitting sarcolemma

Figure 49 (opposite p 146) shows a preparation from a patient with malignant exophthalmos We observe these homogenous masses, this accumulation of metachromatic substance, inside the sarcolemma

Porter. It is inside the muscle cell, is it not?

Asboe-Hansen. It is inside the sarcolemma, extracellularly, because here we see the nuclei in the periphery of the muscular substance at the inside of the "half-moon."





FIGURE 50 *A.* Guinea pig with experimental exophthalmos provoked by injections of thyrotropic hormone *B* Normal gui
Reprinted, by permission, from Asboe-Hansen, G., and Iversen, K Influence of thyrotropic hormone on connective
significance of mucopolysaccharides in experimental exophthalmos. *Acta endocrinol* 8, 90 (1951).



FIGURE 50 *A* Guinea pig with experimental exophthalmos provoked by injections of thyrotropic hormone *B* Normal guinea pig Reprinted, by permission, from Asboe-Hansen, G, and Iversen, K: Influence of thyrotropic hormone on connective tissue, pathogenic significance of mucopolysaccharides in experimental exophthalmos *Acta endocrinol.* 8, 90 (1951)

Porter: Yes, but is it in the cell? The nucleus is in that material, so the material is *really* part of the sarcoplasm, is it not?

Asboe-Hansen: The nucleus is in the muscular substance. In every case, it is placed inside the *mucinous* substance. This observation is apparently not dependent on the basal metabolic rate; we may find the BMR decreased, or it may be increased

Meyer. What muscle is this? The extraocular?

Asboe-Hansen. The quadriceps, and we have preparations from the biceps, too I have even seen it in extraocular muscles, there is a considerable amount of this material in muscle. I think it may interfere with muscular strength. Stained by Hale's method, these structures are blue, indicating acid polysaccharide And stained, for only two minutes, by the McManus-Hotchkiss method, the half-moons become intensely red

In Figure 50 we see two guinea pigs, one normal, and one with exophthalmos after thyrotropin injections Figure 51 shows the half-moon phenomenon Figure 52 shows an electron micrograph of such a muscle fiber. The muscle is fixed with basic lead acetate We see



FIGURE 51 Transversely cut muscle Staining *toluidine blue* Metachromatic half moons between sarcolemma and fibers peripheral nuclei are located at the inside of the half moons Reprinted, by permission from Asboe-Hansen, G Iversen K and Wichmann, R Malignant exophthalmos, muscular changes and thyrotrophin content in serum *Acta endocrinol* 11, 376 (1952)



FIGURE 52 Electron micrograph of half-moon,' transversely cut The structureless substance is accumulated between the sarcolemma and the muscular substance Magnification approximately 14,000 x Reprinted, by permission, from Iversen, K, Asboe-Hansen, G, and Carlsen, F Electron microscopy of certain muscle lesions in patients with pituitary-thyroid disorders *Acta endocrinol* 14, *177 (1953).*

the sarcolemma here, and we may note the fibril structure Between these is the mucinous substance

Figure 53 shows a longitudinal section. It is not very well fixed, but that is because we had to use the basic lead acetate fixation. We observe the cross striations of the muscle, and the sarcolemma to the left

Porter. Is it within the muscle cell, that is, within the sarcolemma?

Asboe-Hansen. It is within the sarcolemma.

Porter. And the sarcolemma runs across the upper left-hand corner of the micrograph, does it not?

Asboe-Hansen. Yes That is right. Even in the electron micrograph

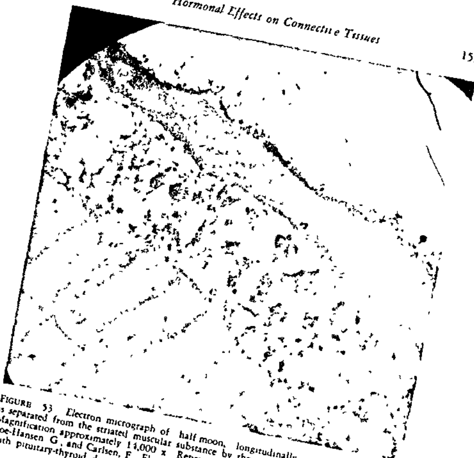
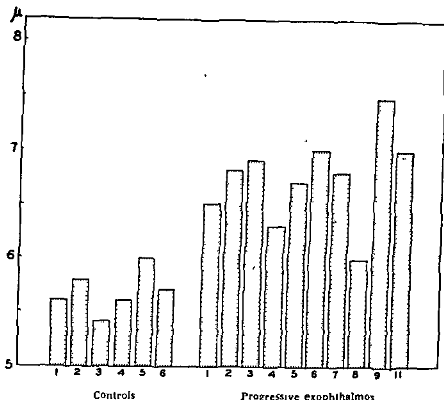


FIGURE 53 Electron micrograph of half moon, longitudinally cut The sarcolemma is separated from the striated muscular substance by the mentioned mucinous substance Magnification approximately 14,000 x Reprinted, by permission, from Iversen K Asboe-Hansen G, and Carlsen, F Electron microscopy of certain muscle lesions in patients with pituitary-thyroid disorders *Acta endocrinol* 14, 177 (1953)

there is no visible membrane between the muscular substance and the mucin On the other hand the demarcation of the half-moons is very sharp in the stained preparations There are no signs indicating that the substance of the half-moon continues into the sarcoplasm And here again I wish to point out that the preparations are fixed in lead subacetate, perhaps that is the reason why the membrane is not demonstrated

Table VI is shown in order to compare the thyrotropin content in blood serum with the half-moon structures, the mucopolysaccharide containing substance within the muscle sarcolemma We found thyrotropic hormone elevated in nine out of ten examined cases

Figure 54 indicates the thyrotropin level in six control patients, and



[illegible]

thalmos, muscular changes and thyrotrophin content in serum *Acta endocrinol* 11, 376 *(1952)*

ten patients with progressive exophthalmos. The thyrotropin levels were significantly elevated in nine out of ten patients One was not particularly elevated These structures cannot be found when formalin-fixed preparations are used, this may be why they have never been noted before

As I said before, we found these changes in the skeletal muscles, in the quadriceps femoris and the biceps brachii In malignant exophthalmos, the retrobulbar tissues have a rather high content of water The neurosurgeons find an extraordinary edema, and report that in extraocular muscles, the normal volume is increased by six or seven times.

The muscular changes were found in ten out of ten examined cases of malignant exophthalmos, in 16 out of 45 cases of thyrotoxicosis, in one out of seven cases of myxedema, and in all five cases of ac-

TABLE VI

[illegible]

Case No	Age	Sex	B M R per cent	Biopsies from Skeletal Muscles: MPS*) within muscle fibres (half moons)	MPS in interstitial connective tissue	Fat within muscle fibres	Thyrotophic hormone content in 1 ml serum (U S P thyrotrophin units)
1	47	♂	+23	numerous	abundant	none	0 0028 (elevated)
2	54	♂	+ 2	numerous	abundant	none	0 0042 (elevated)
3	40	♀	+43	numerous	slight	none	0 0018 (elevated)
4	46	♀	+30	several	abundant	none	0 0021 (elevated)
5	29	♀	− 9	several	slight	none	0 0036 (elevated)
6	51	♀	+18	numerous	abundant	none	0 0054 (elevated)
7	50	♀	+24	numerous	abundant	none	0 0012 (elevated)
8	53	♂	+ 3	several	abundant	none	<0 0015 (not elevated)
9	56	♀	+17	several	abundant	none	0 0104 (elevated)
10	32	♀	− 3	several	slight	none	not examined
11	66	♂	+ 8	not examined	not examined	not examined	0 0054 (elevated)

*) MPS mucopolysaccharides, hyaluronidase sensitive

Reprinted, by permission from Asboe-Hansen G Iversen K and Wichmann R Malignant exophthalmos, muscular changes and thyrotrophin content in serum Acta endocrinol 11, 376 (1952)

comegaly. However, there were none in the 25 patients who did not have pituitary-thyroid disorders We related the findings to the muscular weakness which is often a predominant symptom of these diseases

By collaboration with Dr Wichmann (30), of the State Serum Institute in Copenhagen, the thyrotropin content of these patients' sera was determined. It was done by a biological method, using as an indicator the height of the thyroid cells [illegible] laet [illegible] (31 [illegible] patients with mali[illegible], the thyrotropin level was significantly increased D'Angelo and his associates found their highest thyrotropin values in acromegalic subjects In myxedema, several workers found an increased excretion of thyrotropin in the urine. In thyrotoxicosis the thyrotropin may be inactivated

Sinex. What do you mean by "inactivated" thyrotropin? How do you know *it is there?*

Asboe-Hansen It has no thyroid-stimulating effect The thyroid gland is able to inactivate the thyrotropin hormone

Sinex: How is it determined?

Asboe-Hansen: The thyrotropin may be reactivated by reducing agents, but I do not know of any results with thyrotoxic patients, it

has been done after inactivation *in vitro* by thyroid tissue. We found an increased content of acid mucopolysaccharide in the connective tissue, and a mobilization of fat. These are the essential changes which evidently produce exophthalmos, the cutaneous mucinous edema, and the muscular alterations, perhaps the lipemia in myxedema, too

The effect of thyrotropin on the mucinous system of the connective tissue seems to be in every respect the opposite of the effect of cortisone We performed a few spreading experiments to study the changes in the ground substance These were carried out in exactly the same way as the cortisone experiments, and the results were directly contrary Just as in human myxedema, which is perhaps of fundamentally the same genesis, the direct spreading is very slight, as the diffusion is prevented by the ample content of mucin. On the other hand, the spread of hyaluronidase is highly increased, probably because of the increased quantity of highly polymerized hyaluronic acid (Figure 55).

These effects of the adrenocortical steroids, cortisone and hydrocortisone, as well as the thyroid hormone and the adrenocorticotropic

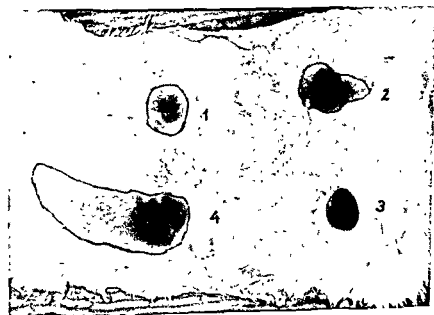


FIGURE 55 Spreading reactions in a white rabbit Indicator India ink ... solution
1 ...onidase
2 ...jected with
3

4 Spreading during thyrotropin administration in ... jected with hyaluronidase

and thyrotropic hormones of the anterior hypophysis, upon the connective tissue, are extremely marked So much so that we are justified in concluding that these hormones have an essential regulating influence on connective tissue in the normal and pathological physiology of the body. Moreover, they have already been utilized in the treatment of connective tissue disorders, despite an almost complete ignorance concerning their pharmacodynamic function Diseases of the skin, the joints, the eyes, and other organs, vascular diseases, infection, wound healing, malignant growths, and metastasis, all have an essential mesenchymal aspect to their pathophysiology, and a good many of them an endocrine aspect as well.

Holbrook Professor Asboe-Hansen, do you think that the mast cell may secrete the mucopolysaccharide, or that it is responsible for its formation?

Asboe-Hansen I investigated a great number of biopsies taken from human subjects, from areas where the chemical and histochemical data indicated that hyaluronic acid was present in large amounts The biopsies were taken from various parts of the body from connective tissue between organs, muscle, and preferably synovial membranes, eyes, umbilical cords, and skin These were the tissues in which I studied the amount and morphology of the mast cells, as well as the ground substance I found a great number, in certain instances a striking number, of mast cells wherever there was hyaluronidase-sensitive metachromatic substance I stained all these preparations with different staining methods My first work was histochemical I used toluidine blue, McManus-Hotchkiss, and Hale staining, and all the routine connective tissue stains The mast cells were increased in number wherever hyaluronic acid was permanently increased

Angevine Did you observe many fibroblasts?

Asboe-Hansen Yes; in certain areas, such as in keloids and tumors, for example, there are many mast cells, but the fibroblasts are not increased in number

Angevine My feeling—and it is just an impression I have, not having studied the mast cells—is that it would be just the opposite From examining large numbers of tissues, the paucity of the mast cell is very noticeable in the specialized areas in which it occurs, in contrast to the large amount of mucopolysaccharides demonstrated histologically

Asboe-Hansen Did you fix it with basic lead acetate?

Angevine Yes, all our material was fixed that way We have examined it, not looking specifically for mast cells, but most of our

studies were done before there was so much interest in them, that is why I asked the question about the synovial membrane

Asboe-Hansen: I agree with you that the number of mast cells does not conform to the amount of metachromatic ground substance, but it does conform to the hyaluronidase-sensitive metachromatic substance, and in skin this is hyaluronic acid.

Meyer The polysaccharide which has been shown to be produced by the mast cells is heparin. This is a polysulfated polysaccharide, while hyaluronic acid is sulfate-free. As you know, heparin is completely resistant to hyaluronidase digestion. While in most tissues, we find simultaneously sulfated and nonsulfated mucopolysaccharides, we have no demonstrable sulfated compounds in synovial fluid or vitreous humor. I believe, also, that the structure of heparin is such that it cannot be related to hyaluronic acid; that is, if the body could desulfate heparin, we would still not obtain hyaluronic acid.

Asboe-Hansen: In skin there is hyaluronic acid, sulfated mucopolysaccaride, and a good many mast cells, but no heparin. In myxedema the amount of mucopolysaccharide is increased, and so is the number of mast cells, but there is still no heparin. In synovial fluid and the vitreous humor, there is no sulfated mucopolysaccharide, and especially no heparin, but plenty of hyaluronic acid and a good many mast cells in the surrounding connective tissue. Heparin and hyaluronic acid are members of the same family· they are both polymeric acid mucopolysaccharides built up by glucuronic acid and glucosamine. The substance within the mast cells has not the same histochemical staining properties as heparin has

Meyer: What do you mean by that?

Asboe-Hansen For example, the McManus-Hotchkiss method reveals this difference.

Meyer. I do not think we ought to get into another discussion of the McManus-Hotchkiss method, but what staining differences do you have in mind?

Asboe-Hansen: Mast cells are stained with the Hotchkiss method, but the method would not stain isolated heparin

Meyer· I would answer by saying that isolated hyaluronic acid is not oxidized by periodic acid.

Asboe-Hansen: Not at all?

Meyer Apparently not. Three different laboratories have agreed on that

Sinex· That has to do with free groups and the degree of polymerization. If we had small residues of hyaluronic acid in the making, would they be expected to be sensitive to it?

Meyer: I think the two end-groups in each molecule would, but since the molecular weight of the polysaccharide is so high this would be well within the limits of the method, experimentally I believe Dr Asboe-Hansen finds really intensive staining with the Hotchkiss-McManus method

How about umbilical cord and skin in different species? As I remember, there are supposed to be large differences in the number of mast cells in the skin of the rat and mouse, as compared to man or pig

Asboe-Hansen. Yes, but these cells are metachromatic They are very slightly granulated, many of them

Meyer. In the mouse or rat skin, are the mast cells degranulated or granulated?

Asboe-Hansen They are granulated

Meyer: And in the pig skin?

Asboe-Hansen: I have not examined pig skin, but I believe in that case they are granulated mast cells, too

Meyer: And in umbilical cord?

Asboe-Hansen. There are granulated mast cells in umbilical cord, especially in the later months, but before that time, we find cells which have the same characteristics as mast cells. They could be mast cells, they contain mucopolysaccharides in the histochemical sense, but many of them are not granulated These are embryonal cells and although they are not granulated, they still might be the same cell type It is a matter of definition whether they are called mast cells or fibroblasts, anyway they are mesenchymal cells

Angetine Is it too farfetched to assume that because the mast cells are in an area of high concentration, perhaps the mucopolysaccharides have been stained metachromatically in that area?

Meyer With regard to heparin I think the evidence is that they do not pick it up, but actually produce it

Angevine I agree with that on heparin

Asboe-Hansen You find no heparin in the skin?

Meyer Heparin has been isolated from rodent skin, which contains a large number of mast cells

Asboe-Hansen Yes, but only in the mast cells And finally, what is heparin?

Meyer We can define heparin chemically or biologically The isolated crystalline barium salt of heparin is a rather well-defined chemical complex Biologically, it is true that many different compounds have heparin activity

Asboe Hansen. The metachromatic ground substance seen around mast cells in normal tissue, and which evidently can be produced by

mast cells after experimental provocation, has no heparin properties. It has never been proved. For example, the ground substance of myxedema In experimental myxedema in an animal, or in a human subject with myxedema, we find enormous amounts of mast cells in the connective tissue and large quantities of metachromatic ground substance. But the connective tissue has no heparin properties.

Meyer: For isolation, we need quite a large amount of tissue, or of mast cells. One would have to attempt a quantitative correlation between numbers of mast cells and heparin activity of the extracts The isolated chondroitin sulfate B fraction of pigskin does have some slight anticoagulant activity, on the order of five to ten per cent of that in the crystalline barium salt of heparin.

Asboe-Hansen: Your opinion is that if there are many cells in tissue, we may chemically extract heparin from that tissue? I agree, because I imagine that the mast cell cytoplasm contains building stones for hyaluronic acid, as well as for heparin

Meyer. And for chondroitin sulfate, too.

Asboe-Hansen. I do not know, I cannot say anything about that, because I could not observe any conformity between the occurrence of chondroitin sulfuric acid and the number of mast cells. For example, in cartilage and in the sclera there are not many mast cells

Meyer: In sclera, the concentration of chondroitin sulfate is small, compared, for example, to cartilage In skin it is much higher.

Asboe-Hansen: Yes, but what about the McManus-Hotchkiss method?

Meyer: I do not know.

Asboe-Hansen. It will not stain chondroitin sulfuric acid, either. Jorpes and his collaborators (32) stated that the mast cells do not contain heparin in the polysulfated form; however, perhaps there is a precursor, as a monosulfated heparin, because the McManus-Hotchkiss method will stain it They contended that a monosulfated heparin would stain, hyaluronic acid will stain too.

Meyer. That will depend on the structure of the compounds.

Asboe-Hansen It was stated that hyaluronic acid would take the stain, and perhaps monosulfuric heparin also, but not chondroitin sulfate or heparin

Meyer: What I said about periodate refers to chemical reactions, not to staining Maybe the histologist using this reaction should stain smears of the compounds in various concentrations, with exactly the same procedures as he uses with the tissues.

Asboe-Hansen I agree Another thing, if we inject hyaluronidase or histamine, we obtain a concourse of mast cells from the vessels

around the vessels, and later on they are seen free in the connective tissue. If we continue to study these tissues, we shall find, after four or five days, or possibly after three, a diffuse metachromatic substance around these groups of mast cells which is susceptible to hyaluronidase, in contrast to heparin, which is not. Chondroitin sulfate is not susceptible either.

Meyer: Chondroitin sulfate of skin is not, but one of the two chondroitin sulfates of other tissues is susceptible

Asboe-Hansen: Yes, but I am speaking only of skin, because we proved that type B of chondroitin sulfuric acid of skin is not susceptible

Meyer: I do not know the explanation for the staining reactions I have also read the statement that the metachromasia of mast cells disappears after treatment with hyaluronidase.

Asboe-Hansen: In freeze-dried tissue, we observed in a few cases, that some of the metachromasia subsided, but not all of it by any means I think it is difficult by histochemical methods, to estimate quantitatively, the degrees of metachromasia I do not think we can do that. But may the mast cells contain sulfuric and nonsulfuric components of this type?

Meyer: Yes, that is possible Immature mast cells have been described which contained no heparin, but presumably another carbohydrate The mature cells are apparently loaded with heparin, and with these strongly metachromatic granules

Asboe-Hansen: Yes, but the fact is that in the synovial membrane, for example, we find a very large number of mast cells In the deeper layers they are heavily granulated and the matrix is faintly metachromatic, but towards the surface we find degranulation and intensely stained metachromatic ground substance The synovial membrane and the synovial fluid contain hyaluronic acid, and the synovia contains no heparin or chondroitin sulfuric acid Together with other observations, I took it as an indication that these mast cells produce hyaluronic acid The same thing is true of the membranes of the eye, I found numerous mast cells in the iris and the ciliary body, and there are no sulfuric polysaccharides in the humors of the eye except hyaluronic acid. Putting these things together, I arrived at the hypothesis that mast cells could be the origin of hyaluronic acid They are the only cell type in the connective tissue containing mucopolysaccharide material

Porter They are the only cells that contain mucopolysaccharide material in the form of granules sufficiently concentrated to be seen

Asboe-Hansen: That is correct

Porter. Do you think of those granules as secretory granules, and

does the identification of mast cells depend on a concentration of those granules?

Asboe-Hansen: Yes

Porter: The mere presence of the cells, of course, does not mean that they are secreting. If we find mast cells with a small number of granules in them, and mucopolysaccharide-staining material around the cell, I would go along with you in the assumption that it came from the cell

Asboe-Hansen: We see that in synovial membrane, skin, and in all connective tissue

Porter: But the point is that the mast cell is heavily loaded with granules, and so might simply be considered as a cell that is ready to secrete, rather than as a cell that is secreting, just as a pancreas cell loaded with granules is able to secrete.

Asboe-Hansen: We find all stages of granulation The cell which is loaded with granules is the cell which is able to secrete, and perhaps ready for secretion. There is some evidence that this mucopolysaccharide substance, which is within the cell limits, is not in the granules but between the granules, and that is why I performed these radiosulfur experiments I should like to know whether the sulfur is within the granules I should also like to study cells that are granulated, and those which are not, and perhaps degranulate the cells experimentally to see whether they take up the sulfur. I found granulated metachromatic mast cells in the tissue, which did not take up radioactive sulfur. But most of them, especially those within the papilloma proper, took up radioactive sulfur very readily The resolution is not high enough to judge the location within the cell

Porter. The suggestion from the radioactive work would be that cortisone interferes with sulfur metabolism

Asboe-Hansen: Yes, and nothing else.

Porter: If that is the case, does one find some alteration in hair formation under cortisone?

Asboe-Hansen: Yes, one does

Porter: Is the hair distinctly different in character?

Holbrook: Yes

Porter: Does it grow as fast?

Asboe-Hansen: It is very puzzling. We can depilate rats by giving ACTH, but if we have patients who have alopecia, they can be treated successfully with ACTH and cortisone, and the hair will grow.

Holbrook: Most women who have been on cortisone for three, four, or five years, will tell you that their hair has never been as lovely before.

Meyer: I wish to point out that we may be discussing two different

things: the effect on sulfate, especially in cartilage which has been shown to be specifically inhibited by cortisone, and the effect on organic sulfur, which is not specifically depressed as far as I know.

Smex: If we wrote down all the chemical steps that are required to synthesize hyaluronic acid, we should have quite a list It is difficult to know where each individual step is carried out

Holbrook: Professor Asboe-Hansen, did you say that the fact the mast cell may be heavily granulated does not necessarily have anything to do with its sulfate uptake?

Asboe-Hansen The autoradiographic experiments indicate that that is right I had really heavily granulated mast cells, which did not take up any trace of sulfate

Holbrook But the effect of the cortisone also was to reduce the intensity of granulation?

Asboe-Hansen. Yes

Holbrook. And with that was there less sulfate uptake?

Asboe-Hansen· Yes

Holbrook But they do not fit together They are not necessarily so?

Asboe-Hansen That is right

Porter We can explain that by saying that in one case there were cells in which the granular population was complete, and in the other cases there were cells which formed granules When the cell synthesizes granules, it incorporates new sulfate

Holbrook Yes, that is right It is a question of dynamics as to which stage it is in.

Asboe-Hansen: The mast cell granules do not necessarily contain sulfur, they may be metachromatic without containing sulfur If we have a preparation from an animal which has been given cortisone, especially in some species such as rats and mice, we shall, in many cases, find some almost or completely normal mast cells around the vessels But the other mast cells free in the connective tissue are degranulated I think that is a feature of some interest considering the origin of these mast cells If we paint with a carcinogenic hydrocarbon, we find, in the first place, huge amounts of mast cells around the vessels, and then all over the tissue From where do they come? Sylvia Bensley (33) found an increase in the number of mast cells after the injection of histamine. She tells us that she has examined small round cells of the connective tissue, becoming granulated, some fibroblasts and endothelial cells become granulated, too, and become mast cells But nobody knows whether there were some degranulated mast cells in those situations which later became granulated We do not know whether there is real heterogeneous regeneration of the mast cells, or if there is a spe-

cial cell type in the connective tissue which can be granulated and degranulated, showing different functional phases.

Holbrook: Has that been done in tissue culture? Can the tissue culture be altered so as to greatly increase or decrease the total number of mast cells in a tissue?

Asboe-Hansen: Paff and Stewart (34) have done some cortisone experiments on tissue cultures of embryonic skin, and find that mast cells are inhibited. Their movements are restricted in cultures where the fibroblastic activity is uninhibited.

Meyer: As mentioned in the Introductory Remarks, we have just finished the first experiments on tissue culture material in collaboration with Drs H Grossfeld, J Decker, and G Godman They grew human skin, and it was astonishing to see the quantity of polysaccharide, presumably hyaluronic acid, elaborated into the culture fluid.

Porter. How about the mast cells?

Meyer: I do not know about mast cells in these tissue cultures

Asboe-Hansen: But they are taken from fetal skin, is that right?

Meyer· Yes.

Asboe-Hansen: The mast cell is quite a dominating cell in the skin connective tissue of embryos Paff used fetal skin, too.

Meyer. May I ask the question the other way around. Where does one find the largest number of mast cells? Apparently they are in the lung and in the liver capsule of some domestic animals, such as the calf, according to Sylvén. I did one experiment on ox lung, and one on the liver capsule of calf or sheep liver. We did not observe any demonstrable quantity of hyaluronic acid from either source We have to assume not only different types of mast cells, but also different functions of mast cells The distinction between fibroblasts and mast cells does not seem to me quite clear.

Asboe-Hansen. In certain animals?

Meyer In slaughterhouse animals, particularly the calf.

Asboe-Hansen: Mast cells are presumed to have other functions than hyaluronic acid production. If we take growing connective tissue, such as wound tissue in the rabbit's ear, we find myriads of mast cells, but few fibroblasts. In the rat, for example, there are not very many granulated mast cells in the wound tissue, but in the surrounding area there may be plenty of them Within the growing tissue, we find many cells which are metachromatic but not granulated. In the rabbit and in the rat, these cells may be identical, even if they appear as different cells by our preparation methods.

Krog· What is your definition of a mast cell?

Asboe-Hansen: A mesenchymal cell which, in its cytoplasm, contains

granules and a substance with mucopolysaccharide characters.

Porter: But the staining of those granules depends upon the existence in them of a substance of mucopolysaccharide type, does it not?

Asboe-Hansen. I did not say that the substance was in the granules I think it is, but I do not know I think that these characteristics are necessary to define a mast cell.

Porter: But are the metachromatic elements the granules themselves?

Asboe-Hansen: The substance between the granules is acidophilic; it would be very strange if an acid mucopolysaccharide were acidophilic.

Meyer. There has been a controversy as to whether the metachromasia of the mast cells is localized in the granules or outside the granules The evidence is in favor of the granules There is also a strongly basic component in the granules which can be stained by acid dyes, since the granules are obviously neutral

Porter: So they can be either one or the other?

Meyer: Just as with nucleic acid in the nucleus.

Porter. Depending on the pH?

Meyer Depending on the pH and on the conditions in which we stain one or the other We can stain in the same location with an acidic or a basic dye

Porter Of course, metachromasia depends on that

Asboe-Hansen. Do you think that the intergranular substance between the granules, being acidophilic, could contain an acid mucopolysaccharide?

Meyer: It could, but Zollinger (35), a Swiss investigator, showed that the acid components of the granules diffuse away into the protoplasma, depending on the treatment

Asboe-Hansen: Yes, if they are put in water.

Meyer Or in saline solution However, if they are not placed in saline solution or in water, they stay put in one localized area.

Asboe-Hansen That is one argument against the work of Julén, Snellman and Sylven (36), but I think these Swedish workers made an important contribution by using differential ultra-centrifugation. They studied the chemical composition of the mast cell constituents, as well as fibroblasts, and other cells

Meyer Other evidence on the correlation between mast cells and heparin may be found in the work of Oliver and Bloom (37) on the mast cell tumors of dogs, and in the work of Jorpes (38). Oliver and Bloom extracted from the tumors, which are solid mast cells, some fifty to a hundred times more heparin than from the liver or lungs of these animals

Asboe-Hansen Yes, but the cytoplasm of the mast cells and the

surrounding ground substance are different materials. Nobody denies that within the cytoplasm there is a heparin-like substance, but the substance outside, the metachromatic substance which seems to come from the mast cells, has no heparin properties. In histophysiological experiments, after intradermal injection of hyaluronidase, I took biopsies day after day and found a crowding of mast cells, and in these regions signs of the new formation of a mucopolysaccharide of the hyaluronic acid type.

Meyer: You think, then that the synovial cells do not produce hyaluronic acid?

Asboe-Hansen: Yes But among the mesothelial cells in the lining there are numerous mast cells.

Meyer: We examined a malignant synovioma, both the original tumor site and liver metastases From both, we obtained large quantities of hyaluronic acid From normal liver, we cannot isolate hyaluronic acid. I do not know the histology of these tumors, but we had no doubt that the tumor cells themselves produced the mucopolysaccharide, just as in the case of human mesothelioma of the pleura and peritoneum, or fowl sarcoma Do you investigate those, by the way?

Asboe-Hansen: No These cells are connective tissue cells

Meyer. But they have no similarity to mast cells.

Asboe-Hansen: There may be no essential difference. They may be the same type of cell.

Meyer: I do not think so I do not know what the similarity would be.

Asboe-Hansen: I have not seen them myself, but they are reported to contain mucin droplets staining red with mucicarmine (39). You do not think that these cells could be related to mast cells? They are neoplastic cells.

Meyer: I could not answer that.

Holbrook: Will they produce heparin?

Meyer: No.

Asboe-Hansen: Bensley's experiment (33), in which granules oc- [illegible] lation- [illegible] t cells [illegible] st cell [illegible] numbers, morphology, and variability in the regions where there is physiological or pathological accumulation of hyaluronic acid. We found a correlation and support for the view that the mast cells produce hyaluronic acid.

Meyer: The possibility exists, of course, that the mast cells synthesize mucopolysaccharides other than heparin. But I do not believe there

is a quantitative relation between mast cells and hyaluronic acid concentration.

Asboe-Hansen: I think it cannot be done

Holbrook: Of course, there is no assurance, Dr Meyer, that the tumor cells you describe would behave as nontumor cells They may not have the same properties at all

Meyer. That is true. It has been reported by Vaubel (40, 41) that synovial mesothelium in tissue culture produces a viscous material

Porter: Mesothelial cells will do the same thing.

Asboe-Hansen Vaubel stained them with neutral red and found them granulated

Porter That is another kind of granule Those are small vacuoles in the cells that take up neutral red, they are not granular components of the type that are found in mast cells

Asboe-Hansen I think it must be very difficult to reach any conclusions regarding these phenomena in tissue culture

Porter I am still interested in this sulfate metabolism versus sulfur metabolism I should like to know whether sulfate could be incorporated in the production of keratin and hair?

Asboe-Hansen. Cystine has been isolated after the injection of sulfate

Meyer Yes, the cystine was not radioactive, as was the sulfate. It would be difficult to see how sulfate could go to sulfhydryl Of course, sulfate can be reduced by bacteria, but the incorporation of sulfate is so rapid, i e, 24 to 48 hours, that I do not think there is time for reduction in significant quantities by the intestinal flora, particularly, in the experiment on S^{35} sulfate, because the sulfate is injected and not ingested

Travell Regarding the mucinous change in the skeletal muscle in patients with thyroid-pituitary disorders, do you have any idea as to where it comes from?

Asboe-Hansen. No The sarcolemma, as far as one knows, is partly connective tissue In thyrotoxicosis, it is very common to find an increased number of nuclei around the muscle fibers The density and pattern of these nuclei are not equal Most pathologists call them lymphocytes There are real lymphocytes in these tissues, but there are mast cells too, in the interstitial connective tissue, especially in the perivascular area, and even around the muscle fibers The reason why these cells are never spoken about is that I think 98 per cent of the pathological laboratories stain their preparations with hematoxylin-

eosin and van Gieson methods only, and fix them in formalin. These faintly granulated cells will not show up in that case. Isn't that right, Dr. Angevine?

Angevine: Yes.

Asboe-Hansen: These routine methods will not reveal the mast cells. I know pathologists who would not understand that the experimental tumors in mice contain millions of mast cells, because they have always seen round cells and thought they were lymphocytes. I think it is quite possible that some of these cells, on or in the sarcolemma, secrete this substance And in these diseases, only, you will find an increased number of nuclei around the muscle fibers.

Meyer. Is that also produced in the experimental guinea pig?

Asboe-Hansen: No, we did not produce it experimentally. But in one series, Dr. Francis Zachariae, of our group at the Laboratory for Connective Tissue Research, University Institute of Medical Anatomy, examined pregnant guinea pigs and found these metachromatic halfmoon structures. Otherwise, in guinea pigs, we did not produce these phenomena with our hormone preparations.

Travell: Are they seen in animals deficient in vitamin E?

Asboe-Hansen: I don't know; I have not examined them.

Travell· In the guinea pig, are there many nuclei around the sarcolemma? I ask because we have reported multiplication of nuclei in guinea pigs during deficiency of antistiffness factors (42, 43), and this is also seen in vitamin E deficiency.

Asboe-Hansen: I have never examined them. I have examined only the patient groups I mentioned, and the controls, which were 25 patients without pituitary-thyroid diseases.

Meyer: You said that the highest incidence was in acromegaly?

Asboe-Hansen: Not the absolutely highest incidence, because we had only five acromegaly patients, and all of them had it. The ten malignant exophthalmos patients we examined all had it, too. But only 16 out of 45 patients with thyrotoxicosis had it, and now we wonder whether we could give any credit to the mechanism. You know, there may be pituitary thyrotoxicosis and thyroigenous thyrotoxicosis.

Travell. You said that one of the seven patients with myxedema showed muscle changes. Did that correlate with the serum thyrotropic levels?

Asboe-Hansen: Only one person had a significantly increased level of serum thyrotropin.

EDITOR'S NOTE. Dr. Travell would like to add the following comment to her remarks at the conference:

This work is very interesting because we are studying a group of patients who are prone to skeletal muscle pain and weakness, and who have hypometabolism, spontaneous creatinuria, and a normal blood protein bound iodine They show some of the features of hypothyroidism, and some of hyperthyroidism

Holbrook: In your animal experimentation work, when you injected thyrotropin, could you produce, in every instance, exophthalmos and the fatty changes, or did it occur in only a certain number of cases?

Asboe-Hansen: They all had it.

Holbrook: Every animal?

Asboe-Hansen: Yes, with some quantitative variations

Holbrook. How long did you inject thyrotropin?

Asboe-Hansen. Maximally, 30 days We examined them from day to day, and on the third day some animals had exophthalmos.

Holbrook: Did you do this in rats and guinea pigs?

Asboe-Hansen Only in guinea pigs

Travell: Did the exophthalmos occur in thyroidectomized animals?

Asboe-Hansen: Yes, and quite as much It is very difficult to judge quantitatively here, but it does occur in thyroidectomized as well as in intact animals, and I think it is because we gave rather high doses of thyrotropin It will overcome the thyroxin produced by the thyroid of the guinea pigs.

Holbrook You say you produce in every guinea pig the exophthalmos and the fatty change Do you mean both effects?

Asboe-Hansen. Yes Within the first day, one observes these livers as I showed them. It occurs in all cases, but the degree of exophthalmos varies somewhat.

Holbrook· When does the exophthalmos develop?

Asboe-Hansen Between the third and thirteenth days

Sinex: Are there changes in the blood lipids?

Asboe-Hansen: Yes, they have lipemia

Travell: Did you attribute the exophthalmos both to the fatty deposit and to the changes in the extraocular muscles?

Asboe-Hansen. We could not exactly combine the accumulation of mucin within the sarcolemma, and the fat mobilization But between the muscle fibers, interstitially, there may be large amounts of metachromatic substance in myxedematous guinea pigs, and in myxedematous human beings, too The mucin evidently interchanges with the fat.

Sinex. Is there anything known about the composition of the lipid which is mobilized?

Asboe-Hansen. Yes, it is mainly neutral fat.

Holbrook: Is this process of exophthalmos and fat mobilization reversible when one stops administering the thyrotropin?

Asboe-Hansen. Yes. Even if we continue, the exophthalmos will regress, perhaps because of antihormones, but we do not know exactly why.

Holbrook. They develop a resistance of some type to the thyrotropin?

Asboe-Hansen: Yes, they do, the fat disappears, and the liver cells degenerate, to a certain degree, if we continue these experiments

Travell. Did you make any observations on the influence of cortisone on the mucin deposits within the muscle? Do cortisone and thyrotropin have opposite effects?

Asboe-Hansen: Some cases of malignant exophthalmos have been treated with cortisone I observed one case of malignant exophthalmos combined with localized myxedema The exophthalmos improved, some regression occurred, and the protrusion decreased. The mucinous substance in the pretibial connective tissue subsided almost totally.

Travell It was in the skin?

Asboe-Hansen: Yes.

Travell How about the muscle?

Asboe-Hansen: We did not examine it, because we could not obtain repeated muscle biopsies from the patient

Holbrook: I did not understand why this subsided.

Asboe-Hansen: The cortisone therapy caused the subsidence of the pathologically increased ground substance.

Sinex. Is there any evidence as to the effect of cortisone and thyrotropin on lipemia, if given together?

Asboe-Hansen: I do not know about that.

Holbrook. If cortisone degranulates the mast cell, does thyrotropin have any effect on the granulations? Does it regranulate the mast cell?

Asboe-Hansen: Thyrotropin causes an accumulation of mast cells in the connective tissue. This phenomenon may be interpreted as a regranulation of degranulated mast cells

Porter: I found the development of mucoproteins in association with the muscle cells interesting, and it seemed to me, from the figures, that the accumulation was mostly intracellular, or should I say, within the cells as well as the sarcolemma. As I understand it, the

sarcolemma is a thin fibrous sheath which is around the cell, and if it is going to be within the sarcolemma and not within the cell, then it has to be between the plasma membrane and the fibrous sheath. From the figures I have the impression there is no plasma membrane between the accumulation of mucoprotein and the myofibrils, which means that it is within the muscle cell.

Asboe-Hansen. That is right But a plasma membrane may be undemonstrable after fixation with lead subacetate

Holbrook: That is, the cross section of the quadriceps is what you are talking about?

Porter: Yes.

Asboe-Hansen: I really think it is outside

Porter: They are outside the contractile elements, but that does not put them outside the cell It makes them part of the sarcoplasm, and this fact becomes fairly important in relation to the claim that the material is derived from mast cells

Asboe-Hansen: The sarcoplasm does not take the metachromatic stain, the Prussian blue, and so on And the mucin even exerts a pressure on the contractile substance The nuclei are observed fairly consistently at the inside of the amorphous substance (Figures 49 opposite p 146 and 51)

Porter: Yes, but you had electron micrographs of the cell in cross section That figure is the longitudinal one Figure 52 is in cross section, and the elements in it are better defined

Asboe-Hansen There is, apparently, no membrane at the inside of the half-moon.

Porter No, there is no membrane There is the sarcolemma Thi becomes sarcoplasm, or at least is continuous with the myofibrils without the interposition of the membrane, so that would make this material intracellular accumulation rather than something produced by cells around the muscle fibrils

Asboe-Hansen Yes But isn't it extraordinary that we always obtain this characteristic localized accumulation?

Porter. What do you mean?

Asboe-Hansen We cannot follow the substance into the sarcoplasm between the myofibrils, and not all the way round the cell, either

Porter There is evidently some compression of myofibrils at this point; they are crowded into one side of the cell, and that somewhat reduces the spaces between them

Angerine: In inflammations, as in gas bacillus infection, we do obtain an increased amount of fluid and protein surrounding the

muscle fibers in the area we are discussing There is *no other place* for it, and it localizes there. It is quite common to see it around the muscle fibrils, so I am inclined to agree with Dr. Porter.

Porter: It would be very interesting to find out what it is and why it accumulates in these cells. But I think I would hesitate to relate it to a mast cell outside the muscle fiber itself. It is quite possible that a variety of cells have the capacity to produce mucoproteins, and among these even muscle cells In Figure 49 (opposite p. 146), as I mentioned earlier, it is so deeply embedded that it would seem to be a nucleus within the muscle cells. The sarcolemma is distinctly separate.

Zweifach Why must it be necessary to visualize a distinct boundary between the living cell and the surrounding tissue? This material may be deposited between the cell surface and what corresponds to its extraneous coat.

Porter: The fibrous sheath of the muscle cell is an extraneous coat which is probably put down by the muscle cell itself.

Asboe-Hansen: Dr. Robb-Smith (44) once published a paper on the sarcolemma and described two layers of it. He could separate them by hyaluronidase.

Porter. That is perfectly reasonable. From the electron micrograph, however, I had the impression that there was no membrane between this deposit of material and the sarcoplasm.

Holbrook: If it is intracellular, isn't it a little difficult to explain why it is always at the pole of the cell, rather than being in the middle of it, or somewhere else?

Porter: I agree.

Asboe-Hansen: You mean that it does not matter whether the cell is inside this area or outside?

Porter. Yes.

Travell: If it were secreted by the sarcolemma sheath, wouldn't you expect to find it all the way around the circumference? If it were secreted inside the sheath, it should move around more freely and be able to coalesce

Porter: It would seem odd to me that it would stay in there at all, if it were enclosed only by the fibrous components of the sarcolemma, because that sheath is obviously porous, whereas the plasma membrane is a thing of another nature.

Gaudino: Wouldn't that be contained within two sheaths of the same membrane?

Porter: I would not expect it to be, but I don't know.

Krog: During sectioning, one very often finds that fragments of

cell components are pushed out of place by the microtome knife This is common with muscle fiber which easily becomes too hard That may explain why a piece of cell, or a nucleus in an unusual location, may be observed in a slide.

Angevine: It is conceivable that if the sarcolemma sheath were stripped from the myoplasm, the nucleus might remain stuck to it

Zweifach: The assumption that diffusion of this material sh occur through the sarcolemma is open to question. Many struct of this type, including the fibrous sheath about nerves, are relatı impermeable even to small electrolytes and water

Porter: Of course, it is an astonishing accumulation of material any case I was just wondering whether it is actually extracellular intracellular.

Zweifach: To my mind, the crescent shape of the deposition wou seem to indicate some localizing factor which is not readily apparen

Porter. Except that it may be isolated, as in a vacuole, and thu kept distinct from the rest of the sarcoplasm

Holbrook: Let us look at Figure 51 for a moment I should like to compare the size Although the detail is not good, there is a little more indication there that it is extracellular

Asboe-Hansen Yes Sometimes it causes an impression in the muscular substance It is excavated Do you see what I mean? It is inside the sarcolemma, and it is just as though it stands under pressure, pressing the muscle in there

Holbrook: Unless we had three dimensions, we could not be certain

Asboe-Hansen. It is seen quite constantly

Zweifach The deposition of the material between the plasma membrane of the cell, and its extraneous coats, may be analogous mechanically to the phenomenon observed in the fertilization of eggs, where a membrane lifts away from the protoplasmic surface

Porter Yes, but you should say that the egg membrane and the fibrous sheath of the muscle cell are two different things

Zweifach: My point there was to indicate that there are biological phenomena in other situations which may shed light on the manner in which this material was deposited

Porter There is no way of settling the question from what we can see here.

Trat ell: Isn't it a different material from that which collects in the interstitial spaces?

Asboe-Hansen: It is stained in the same manner In the histochemical sense, it is the same substance

Angevine: Did you say that there were any mast cells under these conditions?

Asboe-Hansen: I said that in thyrotoxic conditions, and in malignant exophthalmos, too, there are many nuclei.

Angevine: But no more mast cells?

Asboe-Hansen: Granular mast cells, no. But, you see, we have only these preparations to work with.

Gaudino: You mentioned earlier that many of these tissues had an increased amount of water. Do you have any measurements on that?

Asboe-Hansen: No, we have not, but Drs. Ludwig, Boas and Soffer (26) at Mount Sinai Hospital in New York have measured the water content in the retrobulbar tissues and found increased amounts. Hyaluronic acid has a marked water-binding capacity.

Gaudino: Do you mean a large amount of water per unit of dry weight?

Asboe-Hansen: Yes.

Holbrook: Are there marked electrolyte changes in concentration to account for those changes in water?

Asboe-Hansen: Where do you mean?

Holbrook: Take the retrobulbar muscles: you say they become five or six times their volume. Is that due to changes in electrolyte concentration?

Asboe-Hansen: I think nobody knows. All the people who have worked on this problem have taken all the weak parts of the retrobulbar tissue—muscle and glands and loose connective tissue—and just measured water on the whole.

Holbrook: Does it seem to be a simple hydration, or is there some other factor?

Asboe-Hansen: There seems to be hydration, yes. Two neurosurgeons with great experience have told me that the muscles are weak and gelatinous, and the connective tissue and the glandular tissue are very edematous.

Meyer: I think Ludwig, Boas and Soffer published regression curves in which they plotted hexosamine against water content. This indicates that water content parallels the hexosamine. I think this should hold true only in the early stages of exophthalmos, since in the later stages, at least in exophthalmos in man, the tissue becomes more and more fibrotic. It is probable that in the first stage hyaluronic acid is the mucopolysaccharide produced. Then hexosamine and water would parallel each other, while this would no longer be true in the later stages.

Asboe-Hansen: On the other hand, we see spontaneous regression of malignant exophthalmos

Meyer: Yes, but this would be just partial.

Porter. You mentioned that collagen fibers appear to swell in the connective tissue?

Asboe-Hansen: Yes, in myxedematous tissue they appear to swell; they grow basophilic and just burst apart.

Porter Have you any observations on collagen formation under these conditions?

Asboe-Hansen: No

Sinex: Speaking of collagen, I think we ought to mention the recent papers of Bowes and Moss (45) and Heyns and Königsfeld (46), on the end groups of collagen They found that neither collagen nor gelatin have any appreciable N-terminal end groups, that is they were unable to find any large number of free amino groups at the terminal ends of peptide chains in either collagen or gelatin. I have been waiting for somebody to mention collagen as a protein I think that is a significant development in the structure of collagen within the last year It has implications, I think, in the nature of the polymerization which must go on in the formation of the collagen fibril The collagen molecule can come apart in [illegible] discrete groups without the exposure of free amino groups

Porter At the same time, it makes collagen fairly inert, does it [illegible]

Sinex It makes collagen appear fairly inert to the reagent fluoro-dinitrobenzene.

Asboe-Hansen Dr Sinex, you asked me a question concerning [illegible] and thyrotropin The adrenal glands have been shown to [illegible] role in fat mobilization, thus, adrenalectomy completely [illegible] the fat mobilization following the injection of anterior pituitary [illegible] tract. Dr Payne (17) demonstrated this

Sinex: It does it under most other fat-mobilizing conditions

Asboe-Hansen: I think so

Holbrook Did I understand you to say that in the [illegible] mized animal, the injection of thyrotropin would not [illegible] tical extracts re-establishes the normal reaction

Asboe-Hansen Yes, that is right Administration of [illegible]

Travell What does adrenalectomy do to the exoph[illegible]

Asboe Hansen I do not know, I only know of th[illegible] effect But there is some work on the adrenal cortex [illegible] mos Aterman and Greenberg (48) maintain that ex[illegible] be produced by cortisone alone I think this is th[illegible] such results [illegible]

Gaudino: Many years ago, in the course of some experiments I was doing on hypertension in rats, I frequently observed the appearance of exophthalmos when hypertension developed. I began to quantitate this, but did not pursue the investigation any further. I wonder what the explanation might be.

Travell: How was the hypertension produced?

Gaudino: By wrapping one or both kidneys with gauze impregnated with collodion

Holbrook: One method of trying to determine whether we were dealing with thyrotropin, only, or with two or three different hormones, might be to find out whether adrenalectomy interfered with the exophthalmos as well as with the fat changes

Travell: Yes, and try to obtain a divergence.

Holbrook: If it did one thing, and not the other, we would be probably dealing with two rather than one.

Sinex: Whatever condition you choose, the mobilization of fat seems to require the presence of the adrenals.

Asboe-Hansen: We correlated this extraordinary mobilization from depots, and the replacement of this substance with mucin, they seemed to go hand in hand Just when the fat begins to be mobilized, that is, the fat droplets become smaller and the connective tissue septa broader, we observe the mast cells, the lymphocytes, and the metachromatic substance in its place These reactions begin practically instantaneously as a response to the hormone.

Mast cells are very frequently met with in fat tissue, and are just lying around and embracing the fat cells as metachromatic crescents on them It seems possible that the hormonal effect may change the conditions in the tissue, that is, remove the fat and stimulate the production of mucinous metachromatic ground substance, as though an equilibrium were being maintained there.

Krog: What about the definition of the mast cell? A cell is not only defined by its appearance, but also by its morphological structure, its point of origin, how it stains, and so on. Do we have any evidence that can help to explain where the mast cell comes from?

Asboe-Hansen: No. There is much evidence, but nobody knows exactly where it comes from I think most people believe that the fibroblasts and the mast cells have a common histogenous origin I think that is all that can be said about it.

Krog. At what point can it be recognized as a mast cell? When is a fibroblast called a mast cell?

Asboe-Hansen: I do not know exactly whether the fibroblast can turn into a mast cell.

Krog: Does the mast cell show up in the proximity of the blood vessels first?

Asboe-Hansen: Yes In the first place, they are always met with around the vessels If we inject histamine and hyaluronidase, or if we paint with a carcinogenic hydrocarbon, first they are observed along the vessels in large numbers, and thereafter they are seen all over the connective tissue.

Holbrook: You actually suspect, do you not, that the so-called round cell may be the precursor of the mast cell?

Asboe-Hansen: I do not know

Angevine. But the round cell means a lymphocyte I would not think that could be the precursor

Asboe-Hansen The young mesenchymal cell always found around the vessels may be the precursor

Angevine: You think that it may be some modification of the fibroblast? Of course, that is what most people accept now as far as the monocyte is concerned It is a mononuclear cell, some say it can change back and forth, and others say it cannot We have the same argument here, but there is very little evidence by which one might settle it

Asboe-Hansen: It is impressive to see these very numerous mast cells around the vessels in such a short time after one painting with a carcinogen.

Angevine: The aorta contains a considerable amount of acid mucopolysaccharides and mucinous material That is one place, I believe, where we do not see a mast cell

Asboe-Hansen I have observed them there

Angevine Where? In the media?

Asboe-Hansen Yes I have seen them there, too

Angevine I would be surprised to see them in the media One sees long flat cells, but I always considered them to be fibroblasts.

Asboe-Hansen In the early stages of arteriosclerosis I have seen plenty of mast cells.

Angevine We shall have to submit some of our routine autopsy material to this type of examination; that would be a good way to determine it.

Asboe-Hansen But these aortae have been examined 24 hours or more post mortem, is that not right?

Angevine: Yes, that is correct.

Asboe-Hansen If the mast cells are not very heavily granulated, one will not see them.

Angevine Then one has to use fresh material

Asboe-Hansen: Yes.

Krog: It seems possible that if certain cells are transferred into mast cells, thereby increasing the number of the mast cells in the tissue, certain other cells would have to show a decrease in number Is there any evidence for that?

Asboe-Hansen: I think it is rather pointless to try to count these cells One has to take into consideration the number of vessels, and other factors, but especially the number of vessels

Krog: Is any sudden increase in mitosis observed previous to the increase of the number of mast cells in a defined area?

Asboe-Hansen: No, there is practically no increase in mitosis One may see this process in fetal organisms

Krog: Is there an increase in mitosis in other parts of the body prior to local increases in mast cells, suggesting a possible site of their origin?

Asboe-Hansen: No. One may see signs of mitosis, but very seldom Therefore, the theory of heterogeneous regeneration has considerable support; most people believe in that But I think it is astonishing to see, within this very short time, such a lot of mast cells around the vessels. There are not many other cells in these regions. It might be that some cells are passing the vessels from the blood stream On the other hand, it is not probable that the basophilic leukocytes are identical cells The nuclei are different, and their number is too small

Meyer: Is there any relationship between the macrophage and the mast cell; may mast cells ever be mistaken for macrophages?

Asboe-Hansen Yes, there are cases where that has occurred

Meyer Do you think that what some people might call macrophages you would call mast cells?

Asboe-Hansen: I would call it a mast cell only if it has the characteristics which I outlined But I think the completely degranulated mast cell may be misjudged A macrophage could not have the appearance of a mast cell if it had phagocytized material in it But I think a study of nuclei may be of some importance in the differentiation of connective tissue cells It seems to me that the fibroblast nucleus exhibits a structure quite different from that of the mast cell nucleus However, I do not know whether this is constant. On the other hand, in the rat fetus, Holmgren (49) has described two types of mast cells: one has a fibroblast-like nucleus, and the other looks more like a lymphocyte. But these are histological studies by the light microscope. I do not know how they would look in the electron microscope; I should like to see them

Krog: Are there any nucleoli inside the nuclei?

Asboe-Hansen: No, nucleoli are practically never seen. Occasionally, we may see something which looks like a nucleolus, but I think it is just an irregular chromatin distribution of incidental occurrence

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